Research Summary

On

Stability Behaviour of Antihypertensive Drugs and Their Combination with Genotoxicity Study of Degradation Products

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1.0 INTRODUCTION

The chemical stability of pharmaceutical molecules plays an essential role in the safety and efficacy of drugs. Development and validation of stability-indicating analytical procedures, identification of possible degradation products, and determination of degradation pathway, can all be accomplished through forced degradation studies. The forced degradation method enables the generation of degradation impurities in a short period, and from this it is possible to predict the long-term stability of product. Furthermore, stability-indicating methods are capable of detecting changes in the quality, potency, and degradation impurities in pharmaceuticals.

Forced degradation studies are premeditated to generate chemical entities which are related to the parent product and analysing the same by validated analytical method. The different conditions to be studies are various levels of pH solutions, the presence of oxygen and light, elevated temperatures and humidity levels. Not every force degradation study needs to give positive results. The drug/product is declared to be stable and the test is terminated, when no results are obtained under the studied conditions which are equal to those mentioned in accelerated study. Several new drugs possess a good therapeutic window, despite this, drug development has been hindered by concerns over toxicities, mutagenicities, and genotoxicity. It appears that DNA damage is responsible for these adverse effects.

In the present scenario, genotoxicity study and impurity profiling of the drug candidate are one of the most important requirements of new drug approval as well as a submission of a global dossier. Pharmaceutical products must be analysed and controlled for trace-level genotoxic impurities using precise, accurate, and robust analytical methodologies. Moreover, analysis of genotoxic impurities at low ppm levels is challenging because most of them have a reactive and labile nature.

Since the drug substances and drug products will be stored for long durations in the pharmaceutical companies and the pharmacy retail outlets before they are consumed by the patient, it becomes imperative and prudent to study the DPs which could be formed due to various stress factors on the drug substance either during shipment or storage. So to establish the degradation pathways and the DPs we have performed several studies on drug substances and drug products

by applying external stress factors like acid, base, heat, oxidation, light, etc., and characterized the structures of the same. The present thesis covers the drug profiles, UFLC SIAMs development to exclusively separate formed degradants, structure elucidation and characterization of the drugs and DPs, *in silico* drug degradation prediction and toxicity assessment along with a comparison with characterised DPs and confirmation of toxicity of predicted toxic DPs.

2.0 STATEMENT OF THE PROBLEM AND RATIONALE OF THE STUDY

The formation of degradation products is not only limited to, drug excipients interaction, and drug applied chemical exposure interaction, but also there may be the possibility of formation of degradation products of drug-drug incompatibility. The potent drug degradation products may be very toxic so there is a need for the study of drug-drug interaction under forced degradation study and to assess the stability behaviour of combined drug formulation. Also, as per recommended by various international guidelines, there is a need to find out the toxicity of degradation products and confirm it. When a new chemical entity is assessed, toxicological data are used to evaluate its safety and efficacy, which will help to predict its risk/benefit assessment for New Drug Applications (NDAs). Furthermore, the Center for Drug Evaluation and Research (CDER) recommended the genotoxic study for degradation products.

3.0 OBJECTIVE/S:

- To develop and validate the Stability Indicating Assay Method for Active Pharmaceutical Ingredient(s) and Fixed Dose Combinations (FDCs) by HPLC.
- To study the stress degradation behaviour, exploring drug-drug incompatibility in the physical mixture of API(s) and in FDCs.
- To identify and characterize the degradation products (DPs) by instrumental techniques.
- To use *in-silico* tools to predict the possible degradation products and to find out the possible pathway for degradation products.
- To predict the toxicity of formed degradation products by computer software tools like TOPKAT.
- To isolate / synthesis, the toxic degradation product(s).
- To perform/confirm the Genotoxicity of toxic degradation product(s).

4.0 PLAN OF WORK AND RESEARCH METHODOLOGY

- Selection of drugs / drug formulations for project work.
- Extensive literature review, patent search and study of drug profile.
- Procurements of drug and drug products.
- Identification & characterization of drug and drug product.
- Application of the Analytical QbD approach to develop stability indicating assays method for APIs and FDCs
- Forced Degradation Study
  - ➢ Stress conditions are hydrolysis (acid / base influence), light, heat, humidity, and oxidation or even a combination of above cited parameters
  - ➢ The forced degradation study will be performed on three matrices like, API(s), Physical mixture of API(s) and FDCs.
- Preliminary separation studies on the stressed samples and Study of stress degradation behaviour, exploring drug-drug interaction in combination in drug formulation.
  - ✓ Selection of mobile phase to elute drug along with degradants products. (To resolve the degradation products from mixture of stressed sample and separate the degradation products formed under variety of conditions).
  - ✓ Application of QbD approach for method development.
- Final method development and optimization.
- Final Method Validation (Validation as per ICH guidelines.)
- Characterization of degradation products by various instrumental methods like, LC-MS/MS, IR, etc.
- Use of computer software tools to predict the possible degradation products and to find out the possible pathway for degradation products.
- Prediction of toxicity of formed DPs by computer software tools like TOPKAT etc.
- Isolation of degradation product / Synthesis of degradation product.
- Genotoxicity study of formed toxic degradant product.
  - ✓ Ames test assay
- Collection and Interpretation of Data using statistical tools.

5.0 EXPERIMENTAL AND METHODOLOGY

To explore the stability behaviour of antihypertensive drugs, in the present study, three antihypertensive drugs (VAL, NBL, and SAC) and two drug combinations (NBL-VAL and SAC-VAL) were selected. Lupin Pharma and Dr. Reddy's laboratory provided the pure API as gift samples, along with certificates of analysis. The received drug samples were tested and characterised for authentication.

QbD enabled UFLC Method Development

Analytical Target Profile and Critical Analytical Attributes

A critical step in developing QbD-based LC methods was to outline the ATP. The ATP represents the anticipated quality characteristics of the analytical method to ensure its applicability, safety, and quality. To meet the ATP requirements, a variety of CAAs were selected. The parameters of various ATP were summarised in the supplementary file. CAAs were used to measure the characteristics of an analytical method within a specified range or limit to match the anticipated quality of an analytical method. CAAs were selected in this study which significantly influenced the performance of the method, like retention time (Rt), number of theoretical plates (NTPs), and tailing factor (Tf).

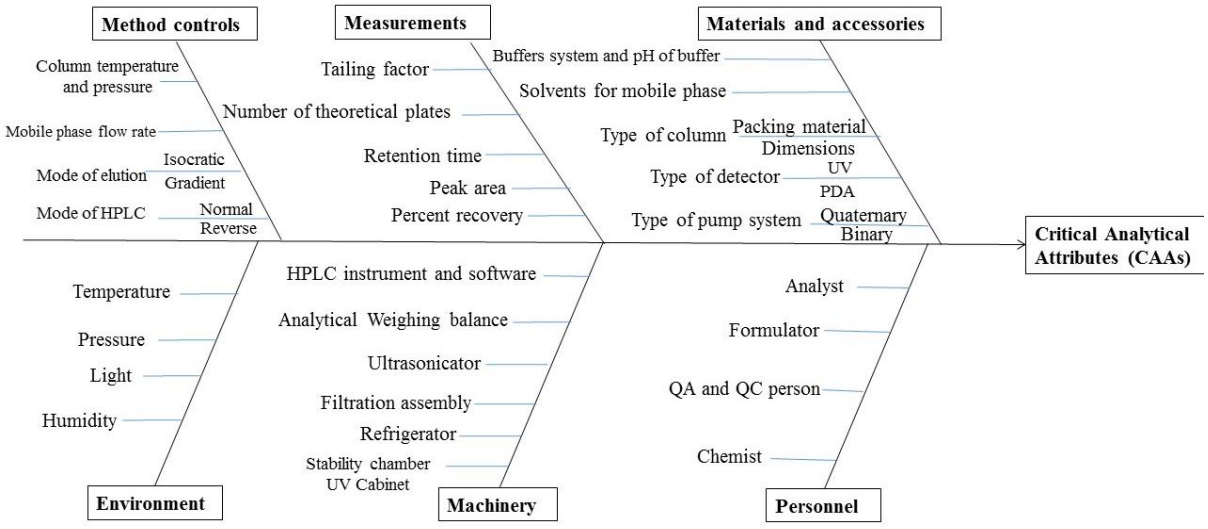


Figure 5.1 Fishbone diagram

Identification of the factors affecting the development of methods based on risk assessment and Taguchi design

A risk assessment study was conducted to determine the likelihood of failures or risks. Using a fishbone diagram, depicted in figure 5.1, the causal relationship between CMAs and CPPs was determined. With five factors varying at two different levels, Taguchi orthogonal array design was applied. CMAs and CPPs and their impacts on CAA methods are identified with this model. In a CMA, solvent % v/v, pH of buffer solution, buffer ratio, and column type were the variables, whereas CPPs were based on flow rate, injection volume, and column type. In all the chromatography runs, these CMAs and CPPs were assessed concerning three CAAs, i.e. Rt, NTPs, and Tf at 10 µg/ml standard solution for all the chromatographic runs. Chromatographic runs were conducted based on the Taguchi design matrix.

Design and optimization of methods using Box-Behnken Design (BBD)

The pH of the buffer solution and the solvent percentage in the mobile phase were selected as CMAs, and the flow rate of the mobile phase as CPPs for further stability indicating method optimization in accordance with the results of the screening techniques. A 3^3 Box-Behnken design was used for the optimization, and 17 experimental runs were carried out as per the design matrix. The experimental data was compared to the indicated CAAs, such as Rt, NTPs, and Tf. By using Design-Expert software, to analyse the experimental data, factor screening was conducted on the experimental data. Figure 5.2-5.7 illustrates the contour plots in 2D and 3D response surface. Furthermore, a second-order quadratic polynomial equation was generated by using the ANOVA F test. (Analysis of Variance)

$$\phi = i0 + r_1A + r_2B + r_3C + r_{12}AB + r_{13}AC + r_{23}BC + r_{11}A^2 + r_{22}B^2 + r_{33}C^2, \ldots$$

Where, ϕ = selected response; i0 = intercept; r_1-r_{33} = regression coefficient, A, B, C = factor investigated.

Multiple parameters were used to evaluate and compare the optimised model provided by the Design, including correlation coefficient, accuracy, and adjusted correlation coefficient. Additionally, 2D contour and 3D response surface plots were generated to analyze the response surface. Moreover, the numerical optimization method was used to predict the optimum analytical condition.

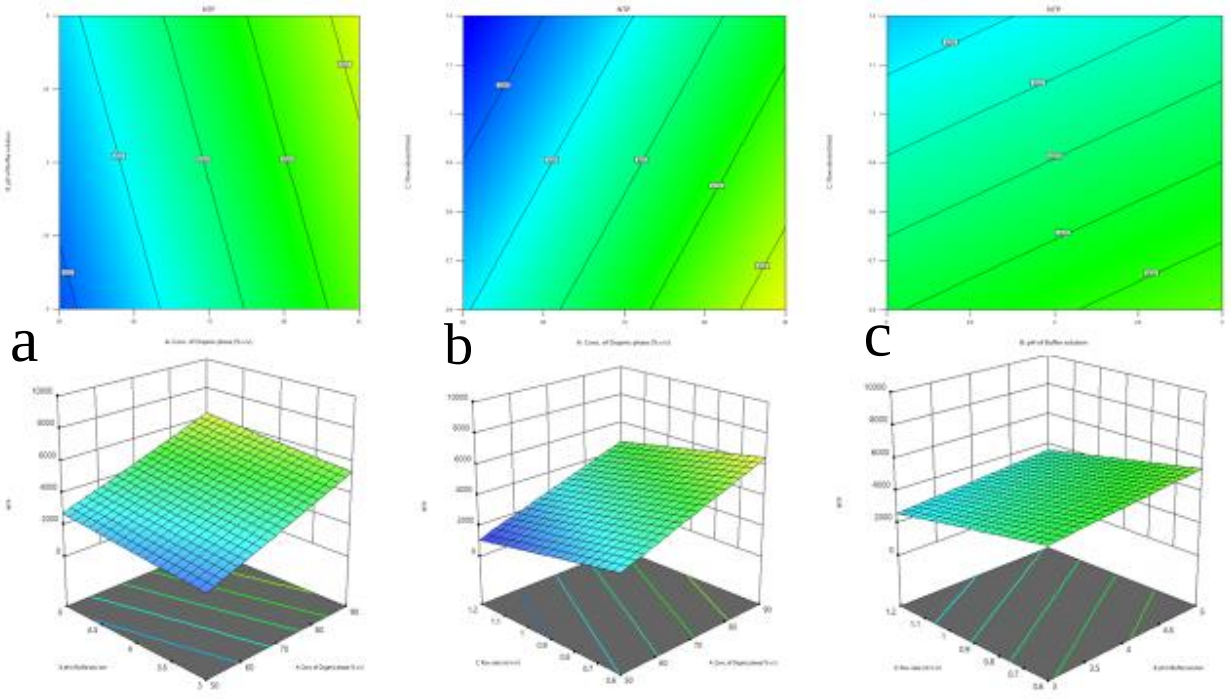


Figure 5.2: 2-D contour plots and 3-D response surface plots showing the influence of CMPs, i.e., % v/v of buffer in the mobile phase (a), pH of the buffer (b), and CPP, i.e., the flow rate of mobile phase (c) on the NTPs as a CAA for VAL

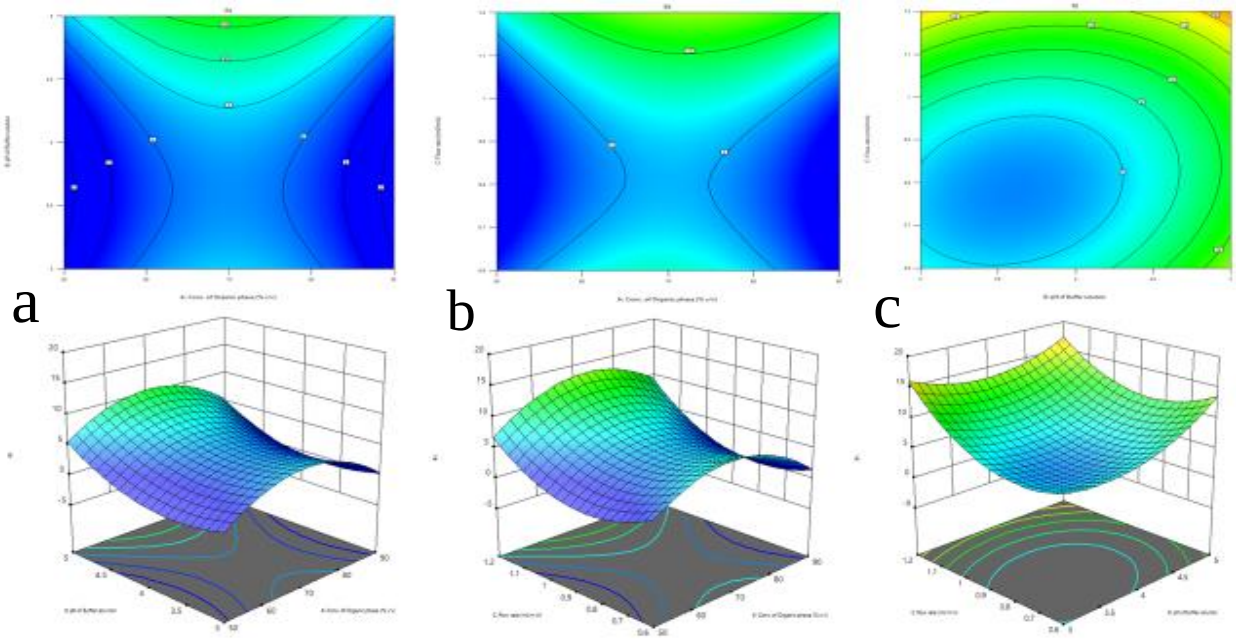


Figure 5.3: 2-D contour plots and 3-D response surface plots showing the influence of CMPs, i.e., % v/v of buffer in the mobile phase (a), pH of the buffer (b), and CPP, i.e., the flow rate of mobile phase (c) on the Rt as a CAA for VAL

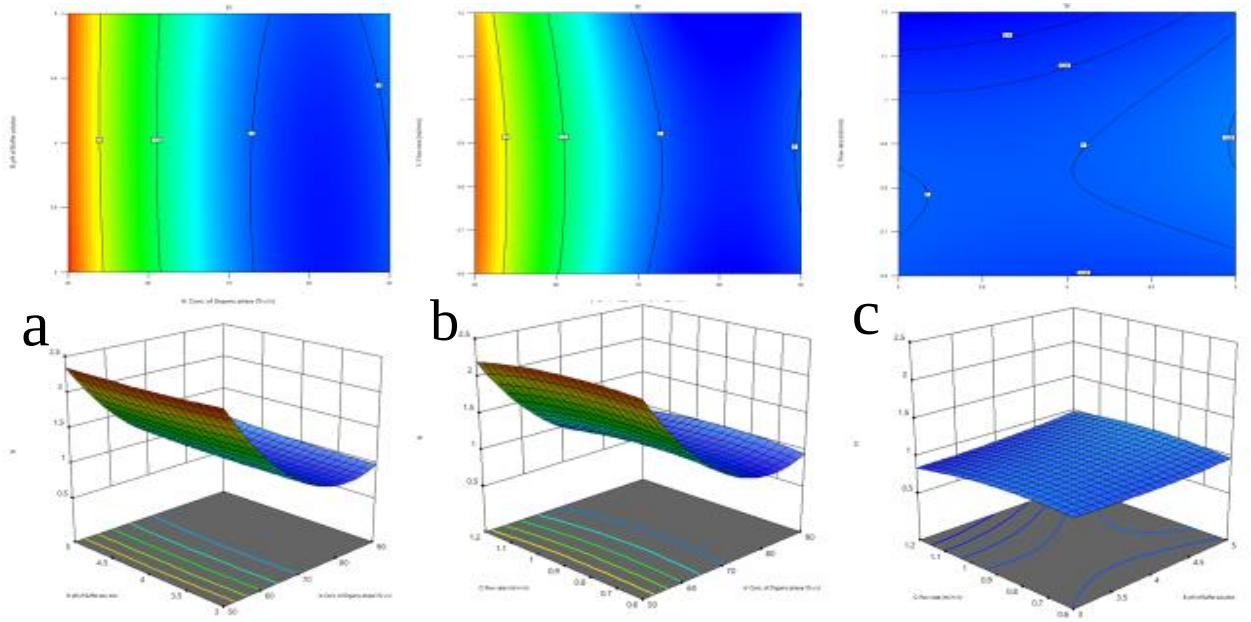


Figure 5.4: 2-D contour plots and 3-D response surface plots showing the influence of CMPs, i.e., % v/v of buffer in the mobile phase (a), pH of the buffer (b), and CPP, i.e., the flow rate of mobile phase (c) on the Tf as a CAA for VAL

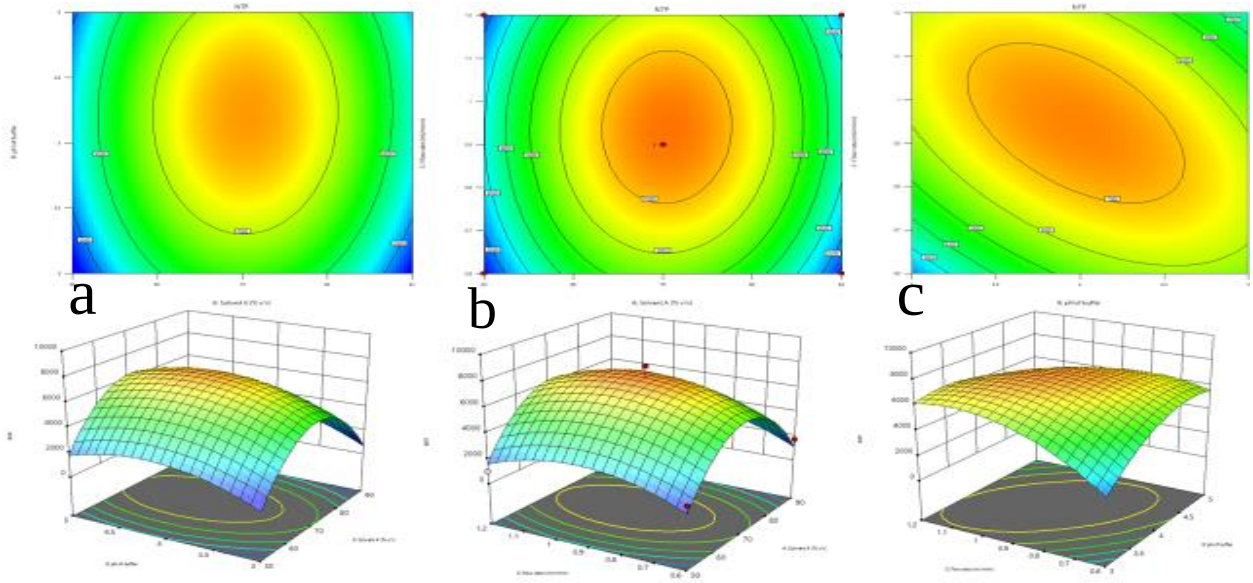


Figure 5.5: 2-D contour plots and 3-D response surface plots showing the influence of CMPs, i.e., % v/v of buffer in the mobile phase (a), pH of the buffer (b), and CPP, i.e., the flow rate of mobile phase (c) on the NTP as a CAA for SAC

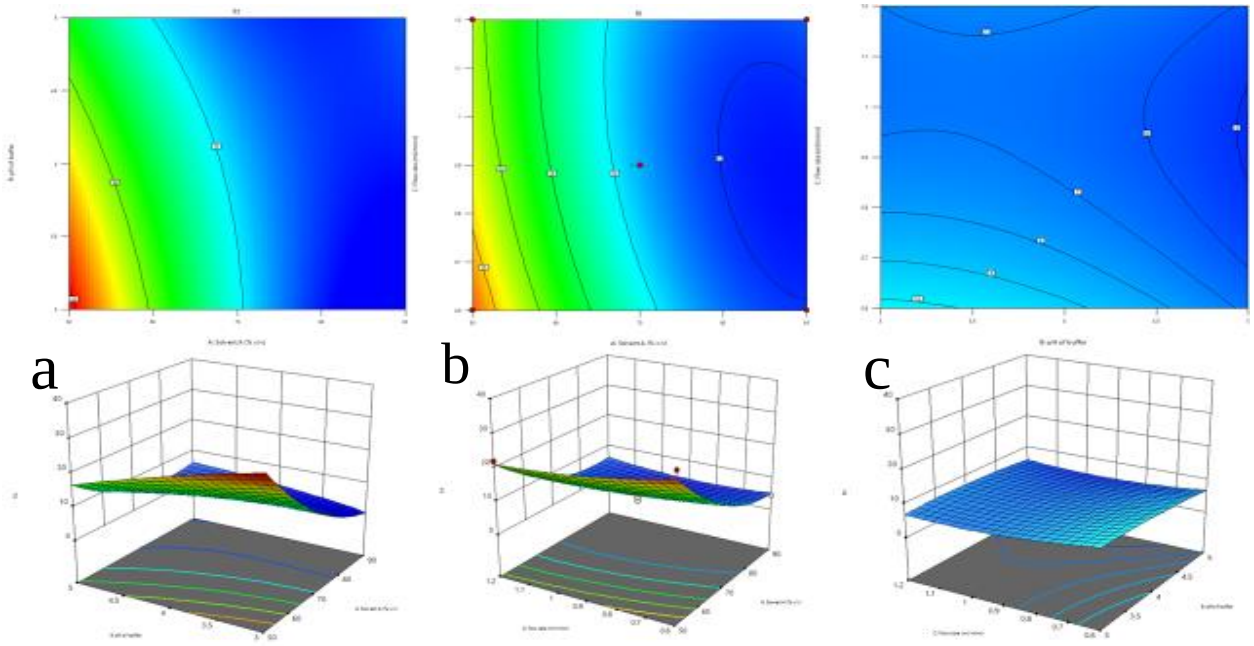


Figure 5.6: 2-D contour plots and 3-D response surface plots showing the influence of CMPs, i.e., % v/v of buffer in the mobile phase (a), pH of the buffer (b), and CPP, i.e., the flow rate of mobile phase (c) on the Rt as a CAA for SAC

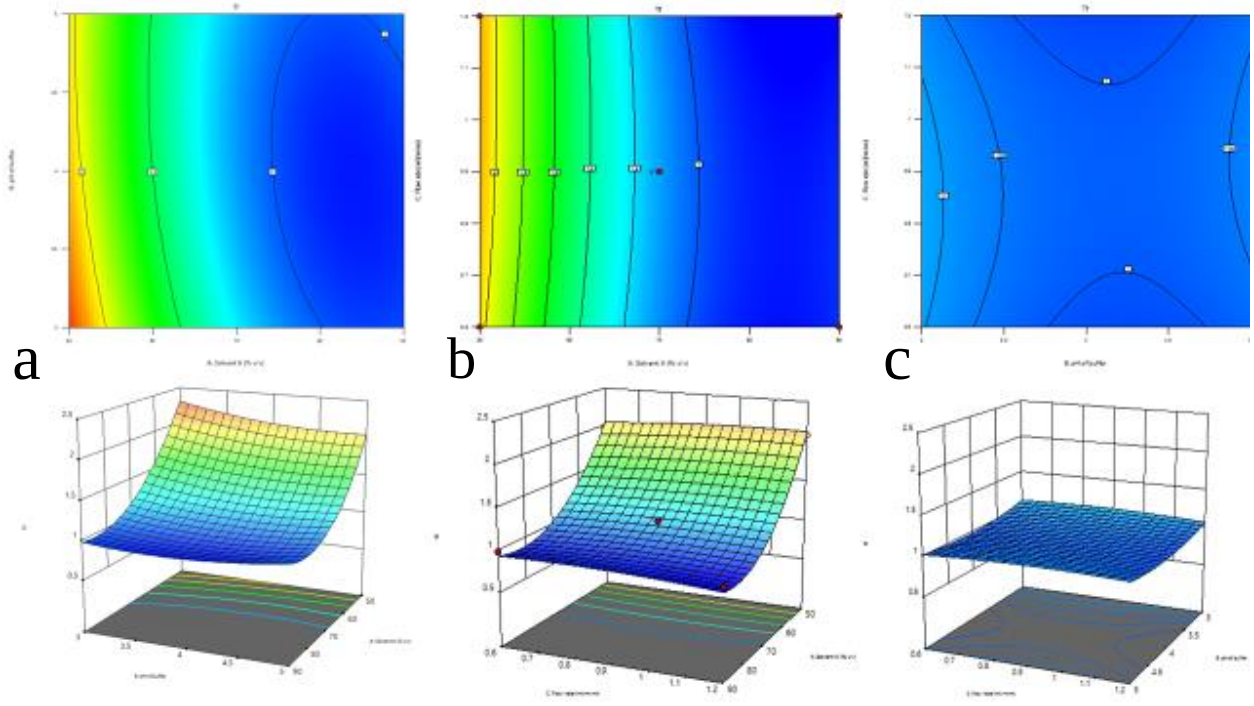


Figure 5.7: 2-D contour plots and 3-D response surface plots showing the influence of CMPs, i.e., % v/v of buffer in the mobile phase (a), pH of the buffer (b), and CPP, i.e., the flow rate of mobile phase (c) on the Tf as a CAA for SAC

Drug degradation prediction

It is challenging to identify all formed degradant at the minute level. The degradants of the potent drug may be toxic in nature, so there is a need to predict the maximum degradants. Zeneth is the only software that can predict drug and drug products' DPs. Zeneth is a software based on expert knowledge that provides quick and accurate predictions for forced degradation. It can be used for the interpretation of structures and degradation pathways likely degradants of DPs or that were detected by MS.

Zeneth can also predict the likely DPs of query compounds, while considering the impact of excipients along with their impurities in a finished pharmaceutical product. Zeneth's comprehensive transformation library is useful for identifying potential degradants, which must be evaluated for mutagenic potential under ICH M7 guidelines. Additionally, the library can assist in determining the chemical structures of degradants detected by MS or other detection methods and predicting likely degradation pathways.

Multiple ICH guidelines (ICH Q1A, Q1B, Q3A, Q3B, M7) emphasise the importance of understanding the impact of degradation and conducting impurity and stability testing, and zeneth's decision support can aid in meeting these requirements. Zeneth's comprehensive transformation library can quickly identify potential degradants, which can then be analysed for toxicity using predictive tools. Zeneth also takes into account reaction conditions specified by ICH guidelines to ensure accurate predictions. The degradation pathways and intrinsic stability of the molecule can be established through stress testing of the API, which can also validate the effectiveness of the analytical methods used. Zeneth presents its predictions clearly and provides supporting evidence related to the degradation pathway.

Degradation prediction for Antihypertensive drugs and their combination

The degradation prediction study for each applied set of conditions was performed with the help of Zeneth software. The degradation prediction study was performed for each matrix and also in the presence of genotoxic impurities. Based on the literature search, there are no available articles on predicting degradation for combined drugs. However, for the first time, an attempt has

been made to predict degradation for combined drugs, including the prediction of degradants for both single API and combined drugs.

Additionally, the impact of genotoxic/mutagenic impurities (NDMA/NDEA) on drug stability and toxic degradant generation was predicted. Through zeneth's prediction, it was discovered that the presence of genotoxic impurities (NDMA/NDEA) in drug samples does not affect the formation rate or number of DPs. However, it was observed in the *in-silico* toxicity study that the presence of genotoxic impurities may trigger the generation of more toxic degradants in comparison to plain drug degradants. The zeneth representative degradation prediction was depicted in figure 5.8. DPs of VAL, NBL, SAC, NBL-VAL, and SCB-VAL were predicted with the help of Zeneth software.

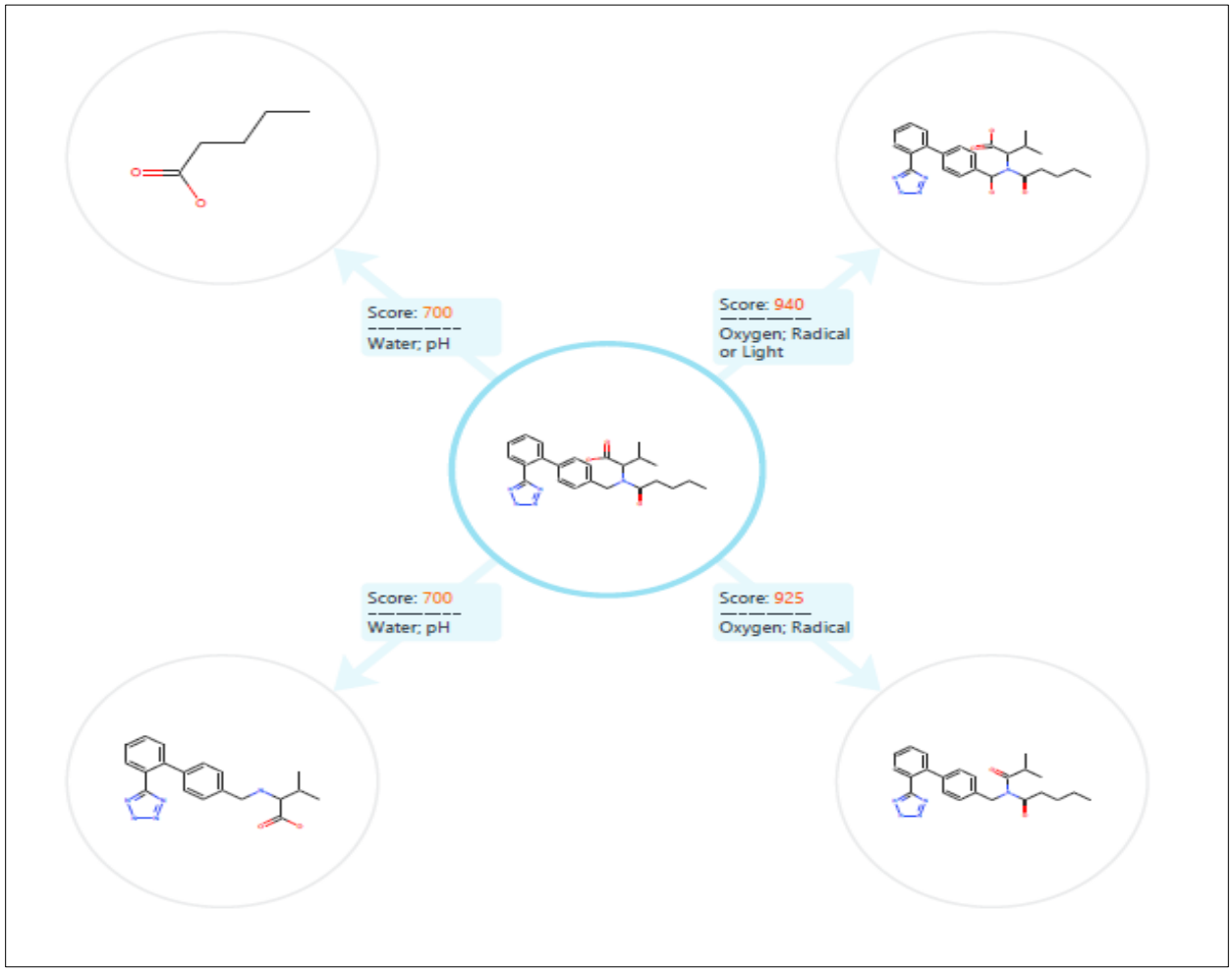


Figure 5.8: Spider diagram of first step acid degradation prediction by Zeneth for VAL

STABILITY INDICATING UFLC METHOD DEVELOPMENT AND VALIDATION FOR VALSARTAN

Standard Solution Preparation:

A precisely weighed 10 mg drug (VAL) was taken into 100 ml volumetric flasks and dissolved in 5 ml MeOH by vigorous shaking and ultra-sonication. To prepare a standard working solution of 100 µg/ml, the diluent is added and the mixture is diluted up to the mark. The appropriate concentration for the working standard solutions is achieved through the serial dilution method. (5-50 µg/ml).

Sample Solution Preparation:

Twenty Tablets of VAL (Diovan 80) were accurately weighed and crushed in a mortar. The powder equivalent to the label claim (VAL, 80 mg) was transferred into a 100 ml volumetric flask. In 10 ml water: MeOH (1:1) solution the powder was dissolved with ultrasonication and a mechanical shaker and with the diluent the mixture was diluted up to the mark. Using the serial dilution method, a working solution was prepared from the prepared stock solution and centrifuged at 7500 rpm for 10 minutes. All prepared standard and sample solutions were filtered by a 0.22µ membrane filter before analysis.

UFLC Method Development

The chromatographic separation was attained by using Shimadzu UFLC, with Kromasil C18 column (250 × 4.6 mm i.d., particle size 5 µm), as the stationary phase, and MeOH: ACN: 20 mM Ammonium acetate buffer (pH 4 adjusted with formic acid) (40:30:30, v/v/v) as mobile phase. The QbD-enabled UFLC method was developed using isocratic mode with a 0.8 ml/min flow rate throughout the chromatographic run. The injection volume was 10 µl. The eluent was monitored by a PDA detector for the quantitation of VAL and its DPs. All determinations were performed at room temperature. Table 5.1.1 contains the optimized chromatographic conditions for VAL. The chromatogram of VAL API was represented in figure 5.1.

Table 5.1: Optimized chromatographic conditions for VAL

Sr. No.	Parameters	Description/ Value
1.	Stationary Phase	Kromasil C18 column- 250 × 4.6 mm with a 5 µm particle size.
2.	Mobile Phase	MeOH: ACN: Ammonium acetate buffer (20 mM, pH adjusted to 4 using formic acid) (40:30:30 v/v/v)
3.	Flow Rate	0.8 ml/min
4.	Detector	SPD-M20A Prominence diode array detector. (PDA)
5.	Elution	Isocratic
6.	Injection Volume	10 µl
7.	Column temperature	Room temperature
8.	Run time	15 min
9.	Diluent	Mobile Phase.
10.	Retention time*	7.884 min
11.	Theoretical Plate*	4913
12.	Tailing Factor*	1.181

*Represents the average of six determinants. (n=6)

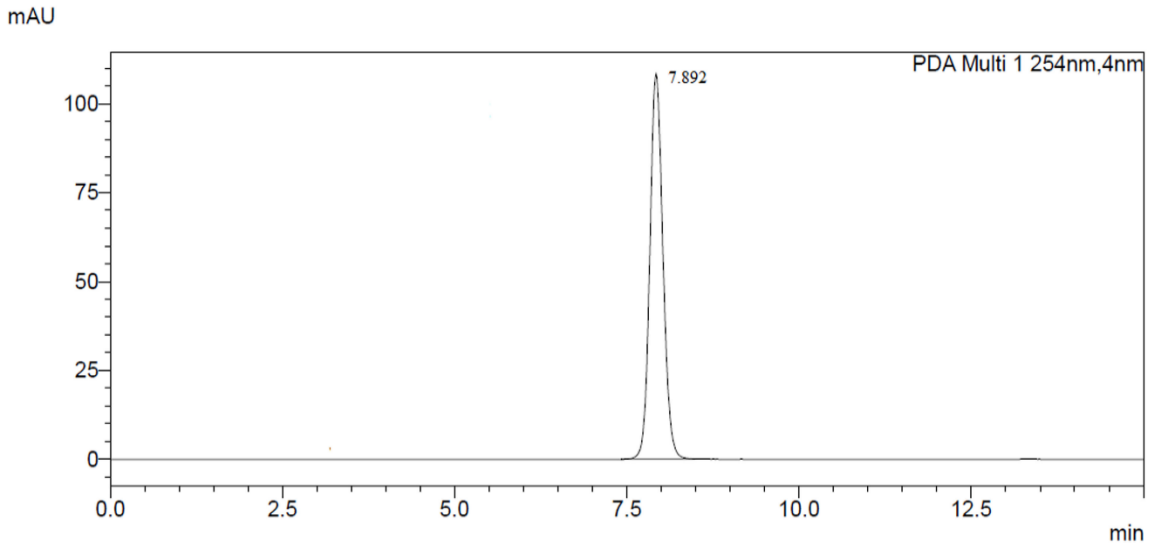


Figure 5.1.1: UFLC chromatogram of VAL API

Stress Studies

As per the ICH guidelines Q1A (R2), stress studies were carried out to exhibit the specificity and stability-indicating nature of the developed UFLC method. The pure drug and drug

products were exposed to different stress conditions like acidic, basic, oxidation, thermal, and photolysis.

Stress Sample Preparation for UFLC Analysis

The exposed samples (after neutralization) for acidic, basic, and oxidative degradation were diluted up to 10 ml with diluent. The photo and thermal stress samples were weighed and diluted with diluent. After dilution, all these prepared stressed samples were filtered with a 0.22 µ syringe filter, and the resulting solution was used to develop a SIM.

Acid Degradation Study

An acid degradation study was performed on VAL in 1 N HCl. 10 mg drug was added in 10 ml HCl solution and kept at room temperature (27°C ± 2) with occasional stirring. Samples were withdrawn at 1 to 7 days and neutralised with NaOH, diluted with diluent, and filtered with a 0.22µ syringe filter prior to injection. The acid degradation chromatogram for VAL was shown in figure 5.1.2. Five DPs were observed in the acid degradation study. (AD_1 - 3.356, AD_2 – 3.621, AD_3 – 6.602, AD_4 – 9.676, AD_5 – 12.349)

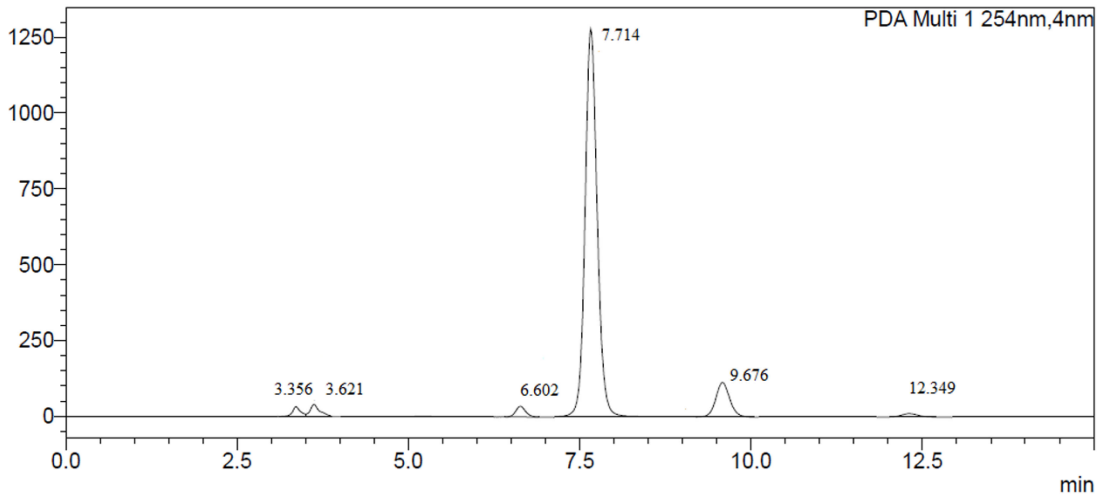


Figure 5.1.2: Acid degradation UFLC chromatogram of VAL

Base Degradation Study

Base degradation studies were performed on VAL in 1 N NaOH. 10 mg of the drug was added, in 10 ml NaOH solution and kept at room temperature (27°C ± 2) with occasional stirring.

Samples were withdrawn at 1 to 7 days and neutralised with HCl and diluted with diluent and filtered with a 0.22µ syringe filter prior to injection. The base degradation chromatogram for VAL was depicted in figure 5.1.3. Four DPs were observed in the base degradation study. (BD_1 – 3.378, BD_2 – 3.682, BD_3 – 6.614, BD_4 – 9.681)

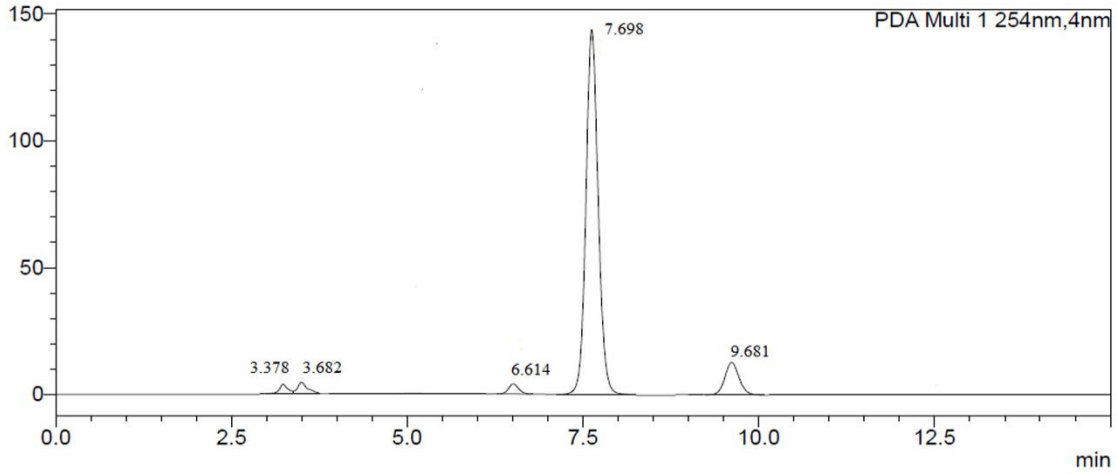


Figure 5.1.3: Base degradation UFLC chromatogram of VAL

Oxidative Degradation Study

Oxidative degradation studies were performed on VAL in 10% H_2O_2. 10 mg drug was added, in 10 ml H_2O_2 solution and kept at room temperature (27°C ± 2) with occasional stirring. Samples were withdrawn at 1 to 7 days, diluted with diluent, and filtered with a 0.22µ syringe filter prior to injection. The oxidative degradation chromatogram for VAL was illustrated in figure 5.1.4. Two DPs were detected in the oxidative degradation study. (OD_1 – 3.854, OD_2 – 6.432)

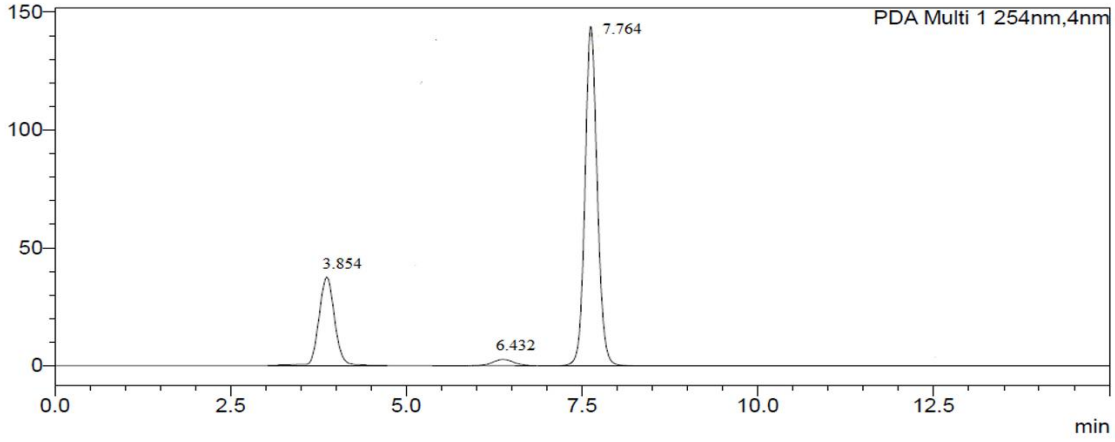


Figure 5.1.4: Oxidative degradation UFLC chromatogram of VAL

Photo-degradation Studies

Photo-degradation studies were performed on VAL. Approximately 100 mg VAL was evenly spread on a glass dish and the layer of sample was less than 2mm thick. The drug was exposed to UV radiation in a UV light chamber. Samples were withdrawn at 1 to 7 days, diluted with diluent, and filtered with a 0.22µ syringe filter prior to injection. The photo-degradation chromatogram for VAL was illustrated in figure 5.1.5. One DP was observed in the photo-degradation study. (PD_1 – 4.326)

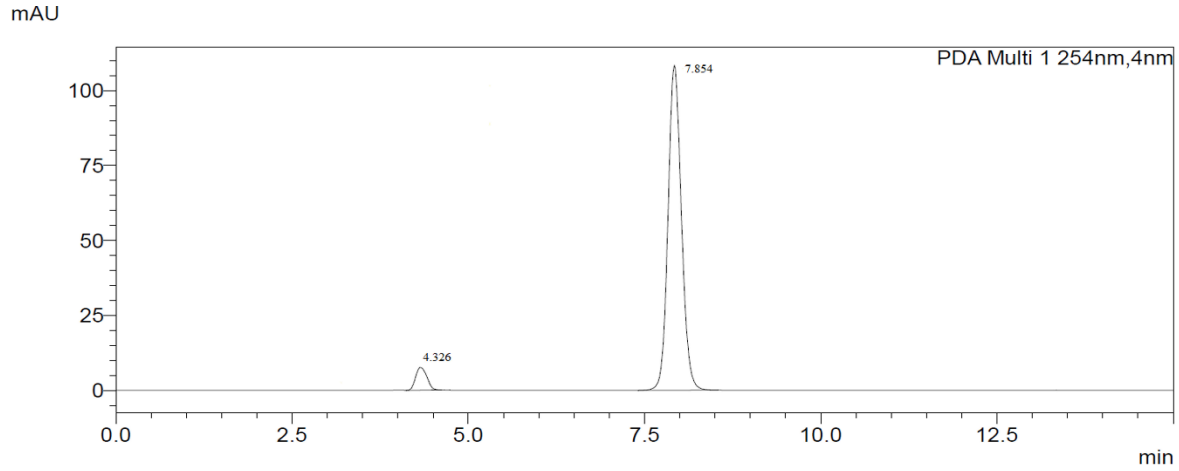


Figure 5.1.5: Photo-degradation UFLC chromatogram of VAL

Thermal Degradation Studies

Thermal Degradation Studies were performed on VAL. Approximately 100 mg of the drug was exposed to a controlled temperature oven at 80°C ± 2°C for 7 days. Samples were withdrawn at 1 to 7 days, diluted with diluent, and filtered with a 0.22µ syringe filter prior to injection. The drug was found to be stable in the thermal degradation study as no DP peak was observed. The thermal degradation chromatogram for VAL was depicted in figure 5.1.6

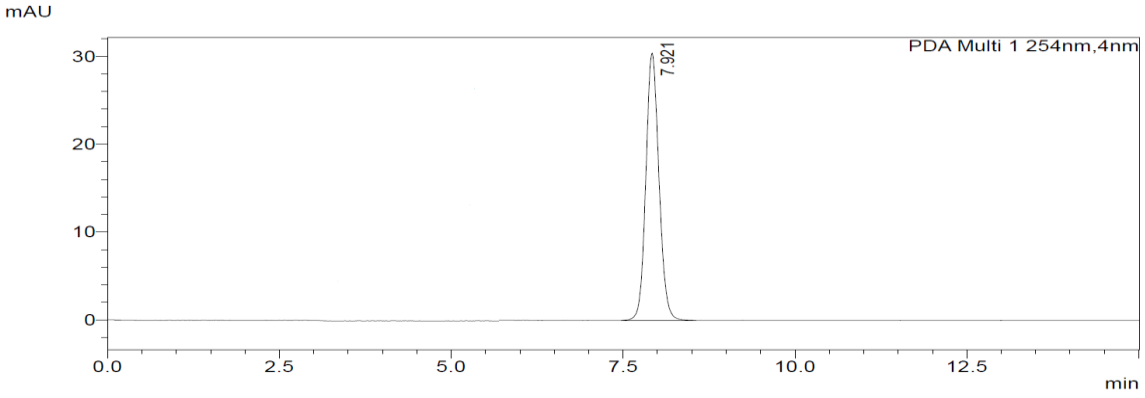


Figure 5.1.6: Thermal degradation UFLC chromatogram of VAL

Method Validation

ICH guidelines were referred for the validation of a developed QbD-enabled UFLC SIM. The validation parameters such as linearity, accuracy, precision, the limit of detection (LOD), the limit of quantification (LOQ), robustness, system suitability testing, and solution stability were confirmed to reveal the anticipated use of the current method.

Linearity: The least square regression method was employed to examine the linearity. A standard calibration curve was created using six different concentrations ranging from 5-50 µg/mL to measure the mean peak area against drug concentration. The developed method demonstrated linear behaviour over the 5-50 µg/mL concentration range with a correlation coefficient of 0.9997, indicating excellent linearity across the study's concentration range. The linearity curve for VAL was represented in figure 5.1.6.

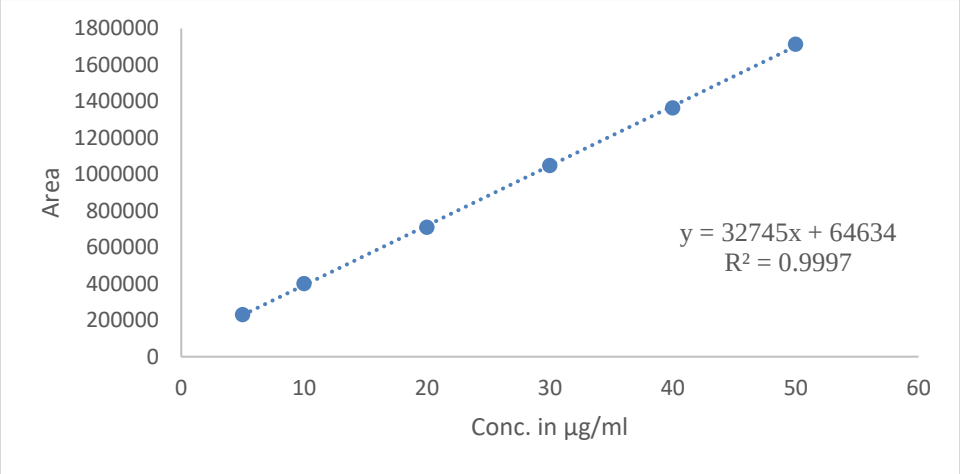


Figure 5.1.7: Linearity curve for VAL

Precision: To conclude the precision of the optimized method, both intermediate precision and repeatability were studied. Repeatability was assessed by examining the samples on the same day. Intermediate precision, on the other hand, was measured by comparing assays performed on different days. Six working standard solutions of 10 µg/mL of VAL were injected on the same day and the next day after sample preparation, and the % RSD of the peak area was calculated. The method demonstrated good precision as evidenced by %RSD values of 0.032 and 0.079 for repeatability and intermediate precision, respectively.

Specificity: Specificity refers to the capability to accurately detect the analyte in the presence of other expected components. To test the specificity of the newly developed method, blank, placebo, and standard solutions were analysed. The chromatograms of the blank and placebo demonstrated no interfering peaks at the drug's Rt. Therefore, the method was deemed to be specific.

Accuracy: The accuracy of the newly developed UFLC method was assessed using recovery experiments, which employed the standard addition method. Known amounts of the standard were added to the sample solution at levels of 80%, 100%, and 120% in triplicate and then analysed using the optimized method. The mean % recovery of the drug was calculated at each level and was found to be within the range of 99.71-100.32%, indicating the method's accuracy. The % RSD between 0.041 and 0.054 indicates a significantly high level of accuracy.

LOD and LOQ: LOD refers to the minimum amount of an analyte in a test sample that can be detected in the solution, whereas the LOQ is the minimum amount of an analyte in a test sample that can be reliably and accurately determined with satisfactory levels of repeatability. LOD and LOQ values of the developed UFLC method for VAL were found to be 0.028 µg/mL and 0.084 µg/mL, respectively.

Robustness: To evaluate the optimized method's robustness, slight adjustments were made to the critical parameters of the method, pH of buffer ((±0.25) such as the flow rate (±0.1 mL/min), and the proportion of organic composition in the mobile phase (±5%). Six replicates of a 10 µg/mL VAL solution were injected and analysed for each condition. The % RSD of peak area for the six replicate injections at all the variable conditions was calculated and found to be less than 2%. From the robustness results, it was concluded that minor alterations in these operational parameters did not result in significant changes in the drug's retention time, theoretical plate count (NLT 2000), or tailing factor (NMT 2). The robustness of the developed HPLC method was demonstrated by the insensitivity of the responses to deliberate slight variations in the method conditions.

System Suitability Testing: The system suitability testing revealed that there were no significant differences in the peak area, NTP, Rt, and Tf of valsartan among six replicates. The %RSD for the

analysed parameters was less than 1%, indicating that the developed analytical method is highly accurate.

Solution Stability: The efficiency of the developed UFLC method was monitored by system suitability tests. Six replicates of the standard drug at the same concentration of 10 µg/mL were injected, and parameters, including NTP, Tf, drug Rt, and % RSD of analyte peak area, were determined. All the results were found to be within acceptable limits.

Results

UFLC Method Development and Optimization

In the development of a product, stability studies play an important role. Stability studies were conducted to analyse the product's quality under different temperatures, light, and humidity conditions. To determine API stability, it is necessary to employ a method that is accurate in indicating stability without interfering with any DPs or impurities. A good separation is desired between VAL and its DPs and/or impurities through the development of stability indicating methods.

Easily available and MS-compatible solvents were used in the development of the UFLC method. Consequently, an attempt was made to develop a straightforward UFLC method using MeOH, ACN, and ammonium acetate buffer. Numerous trials were conducted on a Kromasil C18 column to attain the anticipated separation of the drug and its DPs. The chromatographic conditions were optimized with the help of QbD techniques to develop a UFLC method for the separation of VAL and its DPs. A reversed-phase UFLC method was selected using an ammonium acetate buffer system, compatible with the MS system.

Before analysis, all solvents and buffer solutions were filtered through a 0.22µ membrane filter, sonicated, and degassed. The injection volume was 10 µL, and all chromatographic processing was performed at 254 nm. The optimized column temperature was maintained at room temperature (25-28 ºC). Before injecting the samples, the column was equilibrated with the mobile phase composition.

Assay of VAL

In order to establish the applicability of the developed UFLC method to commercial formulations, the present method was applied for the analysis of the VAL tablet. The average results of the assay were found to be 99.84 ± 0.02. Subsequently, this method can be applied for routine analysis of VAL due to its short analysis time and easy-to-operate technique.

Discussion

Stability Behaviour of Valsartan

The results of stress degradation studies depict that VAL was stable under thermal stress conditions. However, there were five degradants were observed under acidic conditions while four degradants were under basic stress conditions. The detailed study of these degradants revealed that four DPs having closely identical Rt (DP_1 3.356, DP_2 3.621, DP_3 6.602, and DP_4 9.676) are common in both conditions, while the uncommon DP (DP_5 12.349) observed only in acidic condition. Under oxidative stress conditions, two DPs (DP_6 3.854 and DP_7 6.432) were observed. Under the photo-stability study, one DP (DP_8 4.326) was identified. Thus, overall eight DPs were found under various stress conditions. Table 5.1.2 contains the summary of the forced degradation study for VAL.

The stability study samples were analysed using LC-MS/MS to determine the structure of the degradants. The fragmentation pattern and mass spectra were examined to identify the most suitable structure for each degradant. Furthermore, an *in silico* toxicity assessment study was conducted for each structure of the degradants.

Table 5.1.2 Summary of forced degradation study for VAL

Stress condition	No. of degradants observed	Rt of degradants
Acid degradation	5	AD_1 - 3.356, AD_2 – 3.621, AD_3 – 6.602, AD_4 – 9.676, AD_5 – 12.349
Base degradation	4	BD_1 – 3.378, BD_2 – 3.682, BD_3 – 6.614, BD_4 – 9.681
Oxidative degradation	2	OD_1 – 3.854, OD_2 – 6.432
Photo-degradation	1	PD_1 – 4.326
Thermal degradation	0	-

STABILITY INDICATING UFLC METHOD DEVELOPMENT AND VALIDATION FOR NEBIVOLOL HYDROCHLORIDE

Preparation of Standard Solution

A precisely weighed 10 mg drug (NBL) was taken into a clean 100 ml volumetric flask. The drug samples were dissolved in 5 ml MeOH by vigorous shaking and ultrasonication. A standard solution of 100 µg/ml was prepared using the mobile phase as diluent. Using serial dilution, working standard solutions were prepared to achieve an appropriate concentration (5-50 µg/ml).

Preparation of Sample Solution

A clean and dry mortar was used to crush, an accurately weighed twenty tablets (Nebistar 5) of NBL. The powder equivalent to the label claim was (NBL, 5 mg) transferred into a 100 ml volumetric flask. The powder was dissolved in 10 ml water: MeOH (1:1) solution with ultrasonication and a mechanical shaker. Using serial dilution, a working sample solution was prepared by centrifuging the resultant stock solution for 10 minutes at 5000 rpm, in order to achieve a final concentration of 10 ug/ml of NBL, respectively. Prior to analysis, all the standard and sample solutions were filtered through 0.22µ syringe filters.

UFLC Method Development

Several preliminary studies were carried out in order to develop a simple, robust, and stability-indicating UFLC method for evaluating NBL. The chromatographic separation was attained by using Shimadzu UFLC, with Kromasil C18 column (250 × 4.6 mm i.d., particle size 5 µm), as the stationary phase, and ACN: MeOH: 25 mM Ammonium acetate buffer (pH 3.75 adjusted with formic acid) (40:30:30, v/v/v) as mobile phase. The QbD-enabled UFLC method was developed using isocratic mode with a 0.8 ml/min flow rate throughout the chromatographic run. The eluent was monitored by a PDA detector for the quantitation of NBL and its DPs. All determinations were performed at room temperature. The table 5.2.1 contains the optimized chromatographic conditions for NBL. The chromatogram of NBL was depicted in figure 5.2.1.

Table 5.2.1: Optimized Chromatographic conditions for NBL

Sr. No.	Parameters	Description/ Value
1.	Stationary Phase	Kromasil C18 column- 250 × 4.6 mm with a 5 µm particle size.
2.	Mobile Phase	ACN: MeOH: Ammonium acetate buffer (25mM, pH adjusted to 3.75 using formic acid) (40:30:30 v/v/v)
3.	Flow Rate	0.8 ml/min
4.	Detector	SPD-M20A Prominence diode array detector. (PDA)
5.	Elution	Isocratic
6.	Injection Volume	20 µl
7.	Column temperature	Room temperature
8.	Run time	15 min
9.	Diluent	Mobile Phase.
10.	Retention time*	5.864 min
11.	Theoretical Plate*	4430
12.	Tailing Factor*	1.102

*Represents the average of six determinants. (n=6)

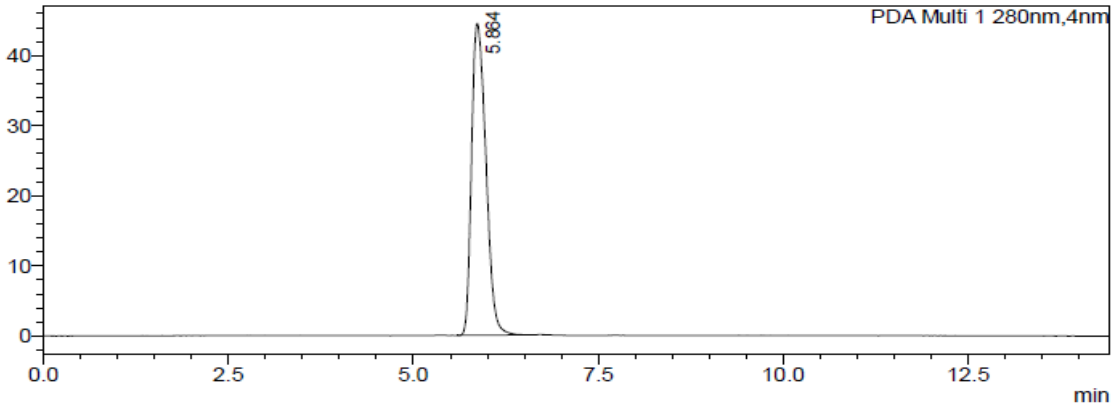


Figure 5.2.1: Chromatogram of NBL

Stress Studies

ICH guidelines Q1A(R2) were followed for the stress tests. The pure drug was exposed to various stress conditions like hydrolysis, photolysis, thermal, and oxidation. The stock solution of the pure drug was prepared in MeOH.

Stress Sample Preparation for UFLC Analysis

The stress samples were diluted with diluent up to 10 ml after exposure to acidic, basic, oxidative, and solution-state photo-degradation conditions. The solid-state samples of thermal and photo stress were weighed suitably and diluted with diluent. Using a 0.22µ syringe filter, all the prepared stressed samples were filtered and used to develop a stability indicating UFLC method.

Acid Degradation Study

An acid degradation study was performed on NBL in 1 N HCl. 10 mg drug was added in 10 ml HCl solution and kept at room temperature (27°C ± 2) with occasional stirring. Samples were withdrawn at 1 to 7 days and neutralised with NaOH and diluted with diluent and filtered prior to injection. The acid degradation chromatogram for NBL was shown in figure 5.2.2. Three DPs (AD_1, AD_2, AD_3) were observed having Rt 3.541, 3.700, and 4.199, respectively.

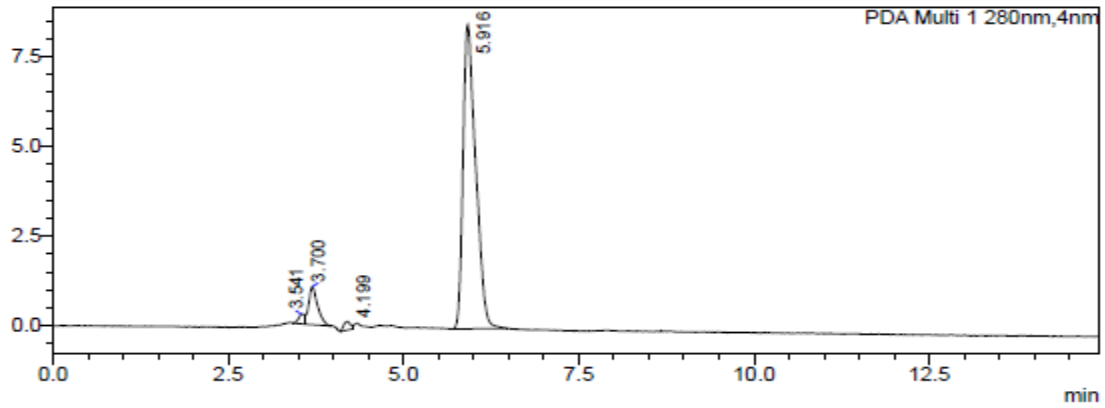


Figure 5.2.2: Acid degradation UFLC chromatogram of NBL

Base Degradation Study

A base degradation study was performed on NBL in 1 N NaOH. 10 mg of the drug was added, in 10 ml NaOH solution and kept at room temperature (27°C ± 2) with occasional stirring. Samples were withdrawn at 1 to 7 days and neutralised with HCl and diluted with diluent and filtered prior to injection. The base degradation chromatogram for NBL was depicted in figure 5.2.3. Two DPs (BD_1 and BD_2) were observed in the base degradation study having Rt 3.693, and 4.185 respectively.

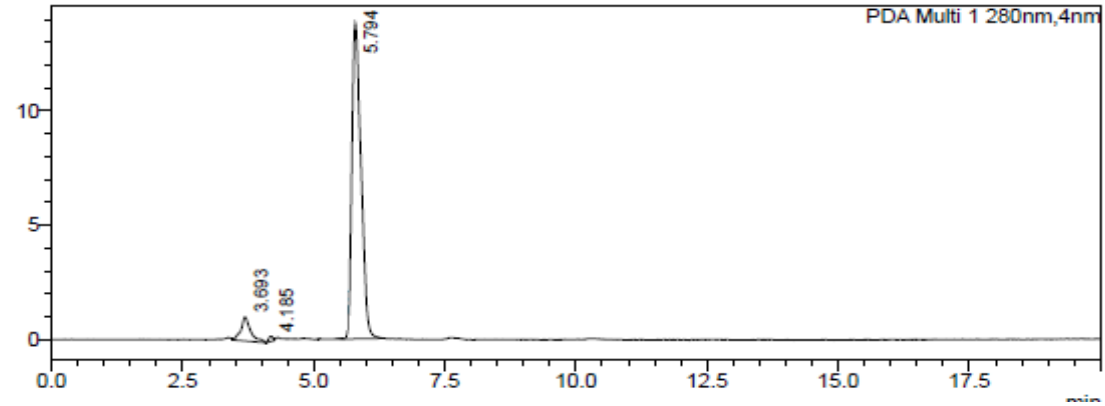


Figure 5.2.3: Base degradation UFLC chromatogram of NBL

Oxidative Degradation Study

An oxidative degradation study was performed on NBL in 10% H_2O_2. 10 mg drug was added, in 10 ml H_2O_2 solution and kept at room temperature (27°C ± 2) with occasional stirring. Samples were withdrawn at 1 to 7 days, diluted with diluent, and filtered prior to injection. The oxidative degradation chromatogram for NBL was illustrated in figure 5.2.4. One DP (OD_1) was detected having Rt at 3.709.

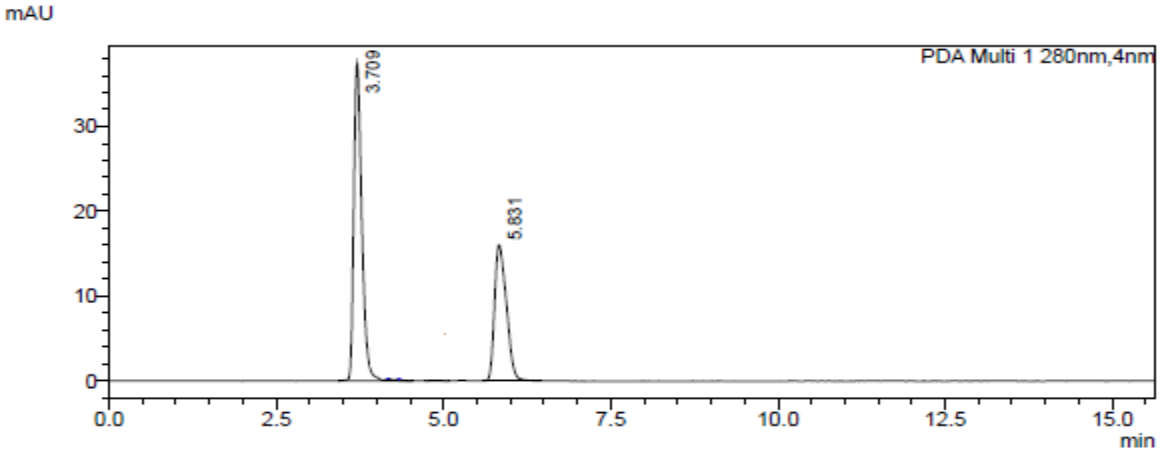


Figure 5.2.4: Oxidative degradation UFLC chromatogram of NBL

Thermal Degradation Study

A thermal degradation study was performed on NBL. Approximately 100 mg of the drug was exposed to a controlled temperature oven at 80 °C ± 2 °C for 7 days. Samples were withdrawn at 1 to 7 days, diluted with diluent, and filtered prior to injection. No DP peak was observed on the chromatogram. The absence of DP in thermal degradation studies (figure 5.2.5) demonstrates that the drug remained stable to heat.

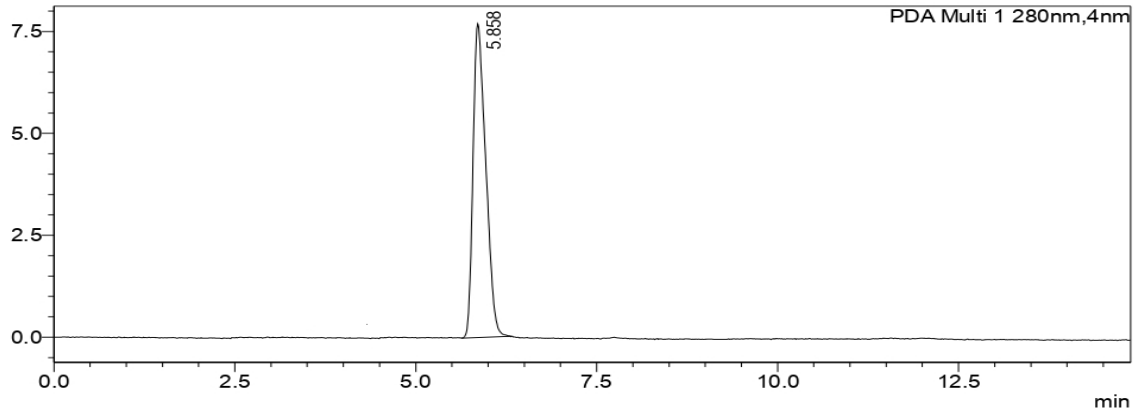


Figure 5.2.5: Thermal degradation UFLC chromatogram of NBL

Photo-degradation Study

The photo-degradation Study was performed on the NBL drug. Approximately 100 mg NBL was spread evenly on a glass dish in a layer that was less than 2 mm thick. The drug was exposed to UV radiation in a UV light chamber. Samples were withdrawn at 1 to 7 days, diluted with diluent, and filtered prior to injection. The absence of DP in photo-degradation studies (figure 5.2.6) indicates that the drug is stable under light exposure.

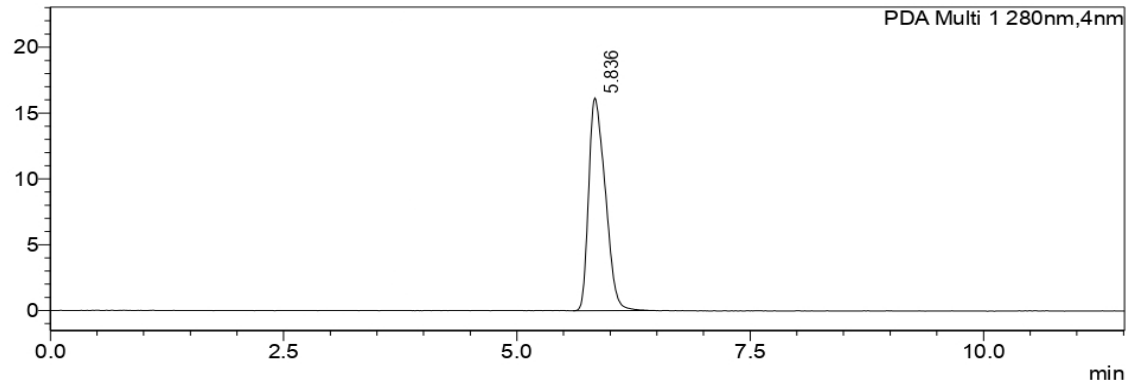


Figure 5.2.6: Photo-degradation UFLC chromatogram of NBL

Method Validation

The developed QbD-enabled UFLC SIM was validated according to the ICH guidelines.

Linearity: A standard calibration curve was then created using six different concentrations ranging from 5-50 µg/mL to measure the mean peak area against drug concentration. The developed method demonstrated linear behaviour over the 5-50 µg/mL concentration range with a correlation coefficient of 0.9996, indicating excellent linearity across the study's concentration range. The linearity curve for NBL was represented in figure 5.2.5.

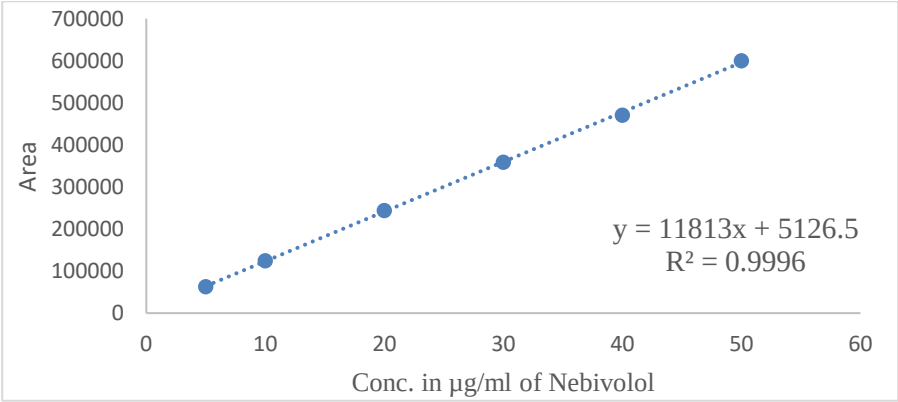


Figure 5.2.7: Linearity curve for NBL

Precision: To conclude the precision of the optimized method, both intermediate precision and repeatability were studied. Repeatability was assessed by examining the samples on the same day. Intermediate precision, on the other hand, was measured by comparing assays performed on different days. Six working standard solutions of 10 µg/mL of NBL were injected on the same day and the next day after sample preparation, and the % RSD of the peak area was calculated. The method demonstrated good precision as evidenced by %RSD values of 0.042 and 0.084 for repeatability and intermediate precision, respectively.

Specificity: Specificity refers to the capability to accurately detect the analyte in the presence of other expected components. To test the specificity of the newly developed method, blank, placebo, and standard solutions were analysed. The chromatograms of the blank and placebo demonstrated no interfering peaks at the drug's Rt. Therefore, the method was deemed to be specific.

Accuracy: The accuracy of the newly developed UFLC method was assessed using recovery experiments, which employed the standard addition method. Known amounts of the standard were added to the sample solution at levels of 80%, 100%, and 120% in triplicate and then analysed using the optimized method. The mean % recovery of the drug was calculated at each level and was found to be within the range of 99.31-100.18%, indicating the method's accuracy. The % RSD between 0.038 and 0.064 indicates the significantly high level of accuracy.

LOD and LOQ: sample that can be reliably and accurately determined with satisfactory levels of repeatability. LOD and LOQ values of the developed UFLC method were found to be 0.088 µg/mL and 0.277 respectively.

Robustness: To evaluate the optimized method's robustness, slight adjustments were made to the critical parameters of the method, pH of buffer (±0.25) such as the flow rate (±0.1 mL/min), and the proportion of organic composition in the mobile phase (±5%). Six replicates of a 10 µg/mL NBL solution were injected and analysed for each condition. The % RSD of peak area for the six replicate injections at all the variable conditions was calculated and found to be less than 2%. From the robustness results, it was concluded that minor alterations in these operational parameters did not result in significant changes in the drug's retention time, theoretical plate count (NLT 2000),

or tailing factor (NMT 2). The robustness of the developed HPLC method was demonstrated by the insensitivity of the responses to deliberate slight variations in the method conditions.

System Suitability Testing: The system suitability testing revealed that there were no significant differences in the peak area, NTP, Rt, and Tf of NBL among six replicates. The %RSD for the analysed parameters was less than 1%, indicating that the developed analytical method is highly accurate.

Solution Stability: The efficiency of the developed UFLC method was monitored by system suitability tests. Six replicates of the standard drug at the same concentration of 10 µg/mL were injected, and parameters, including NTP, Tf, drug Rt, and % RSD of analyte peak area, were determined. All the results were found to be within acceptable limits.

Result

A cost-effective and straightforward UFLC method was developed by using ACN, MeOH, and Ammonium acetate buffer (25 mM, pH 3.75 adjusted with formic acid) as the mobile phase. To achieve the desired separation of the drug sample and formed DPs, various experiments were conducted on a Kromasil C18 column. Forced degradation studies were performed on NBL, to assess the stability behaviour of the drug during the forced degradation study.

Assay of NBL

To establish the applicability of the developed UFLC method to commercial formulations, the validated method was applied for the analysis of the NBL tablet. The average results of the assay were found to be 99.24 ± 0.02. Subsequently, this method can be applied for routine analysis of NBL due to its short analysis time and easy-to-operate technique.

Discussion

Stability behaviour of NBL

The results of stress degradation studies depict that NBL was stable under thermal and photo-stress conditions. However, three degradants were observed under acidic conditions (DP_1

3.541, DP_2 3.700, and DP_3 4.199) while two degradants were in basic stress conditions (DP_2 3.693, and DP_3 4.185). The detailed study of these degradants revealed that two DPs having nearly same retention time are common in both conditions, while the extra DP was observed only in acidic condition (DP_1 3.541). Under oxidative stress conditions, one DP (DP_4 3.709) was detected. As a result, four DPs were identified for NBL when exposed to different stress conditions.

STABILITY INDICATING UFLC METHOD DEVELOPMENT AND VALIDATION FOR NEBIVOLOL-VALSARTAN

Standard Solution Preparation

A precisely weighed 10 mg drug (NBL and VAL) was transferred into a clean 100 ml volumetric flask. The drug samples were dissolved in separate volumetric flasks in 5 ml MeOH by vigorous shaking and ultrasonication. A standard solution of 100 µg/ml was prepared using the mobile phase as diluent. Using serial dilution, working standard solutions were prepared to achieve an appropriate concentration (5-50 µg/ml).

Sample Solution Preparation

A clean and dry mortar was used to crush, an accurately weighed twenty tablets of NBL-VAL (Nebicard V). The powder equivalent to the label claim was (NBL-VAL, 5/80 mg) transferred into a 100 ml volumetric flask. The powder was dissolved in 10 ml water: MeOH (1:1) solution with ultrasonication and a mechanical shaker. Using serial dilution, a working sample solution was prepared by centrifuging the resultant stock solution for 10 minutes at 5000 rpm, in order to achieve a final concentration of 5/80 ug/ml of NBL-VAL, respectively. Prior to analysis, all the standard and sample solutions were filtered through 0.22µ syringe filters.

UFLC Method Development for NBL-VAL

The chromatographic separation was attained by using Shimadzu UFLC, with Kromasil C18 column (250 × 4.6 mm i.d., particle size 5 µm), as the stationary phase, and MeOH: ACN: 20 mM Ammonium acetate buffer (pH 3.75 adjusted with formic acid) (40:30:30, v/v/v) as mobile phase. The UFLC method was developed using isocratic mode with a 0.8 ml/min flow rate throughout the chromatographic run. The injection volume was 20 ul. The eluent was monitored

by a PDA detector for the quantitation of NBL-VAL and its DPs. All determinations were performed at room temperature. Table 5.3.1 contains the optimized chromatographic conditions for a physical mixture of NBL-VAL. The resultant chromatogram of NBL-VAL was depicted in figure 5.3.1.

Table 5.3.1: Optimised chromatographic conditions of developed UFLC method for NBL-VAL

Sr. No.	**Parameters**	**Description/ Value**	
1.	Stationary Phase	Kromasil C18 column- 250 × 4.6 mm with a 5 µm particle size.	
2.	Mobile Phase	MeOH: ACN: Ammonium acetate buffer (20 mM, pH adjusted to 3.75 using formic acid) (40:30:30 v/v/v)	
3.	Flow Rate	0.8 ml/min	
4.	Detector	SPD-M20A Prominence diode array detector.	
5.	Elution	Isocratic	
6.	Injection Volume	20 µl	
7.	Column temperature	Room temperature	
8.	Run time	20 min	
9.	Diluent	Mobile Phase.	
10	Retention time*	NBL- 5.894	Val- 7.793
11	NTP*	NBL- 4350	Val- 4174
12	Tailing factor*	NBL- 1.261	Val- 1.150
13	Resolution	5.784	

*= value represents the average of six determinates (n=6)

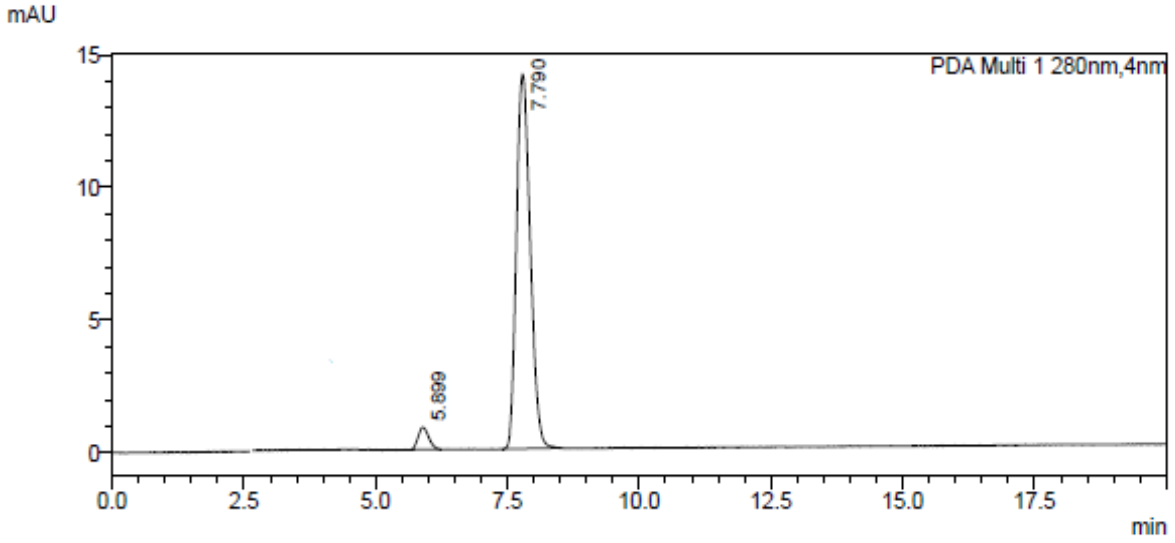


Figure 5.3.1: UFLC Chromatogram for NBL-VAL physical mixture (1:16)

Force Degradation Studies on Physical Mixture of NBL-VAL

ICH guidelines Q1A(R2) were followed for the stress tests. The physical mixture was exposed to various stress conditions like hydrolysis, photolysis, thermal, and oxidation. The stock solution of pure drug and the physical mixture of the drug was prepared in water: MeOH (1:1 v/v). The stability of both NBL-VAL drugs was evaluated through force degradation studies, using a physical mixture of the drugs in two different ratios. The first ratio, 1:16 (5 mg: 80mg), was based on the marked FDCs. The second ratio, 1:1, was used to investigate the stability behaviour of the drugs with a difference in quantity.

Acid Degradation Studies

Acid degradation studies were performed on the physical mixture of NBL-VAL in 1 N HCl. The mixture of drugs in 1:1 and 1:16 ratio was added, separately, in 50 ml HCl solution and kept at room temperature (27°C ± 2) with occasional stirring. Samples were withdrawn at 1 to 7 days and neutralised with NaOH and diluted with diluent and filtered prior to injection. The acid degradation chromatogram of NBL-VAL was depicted in figure 5.3.2. Five DPs were observed in the acid degradation study (1:1).

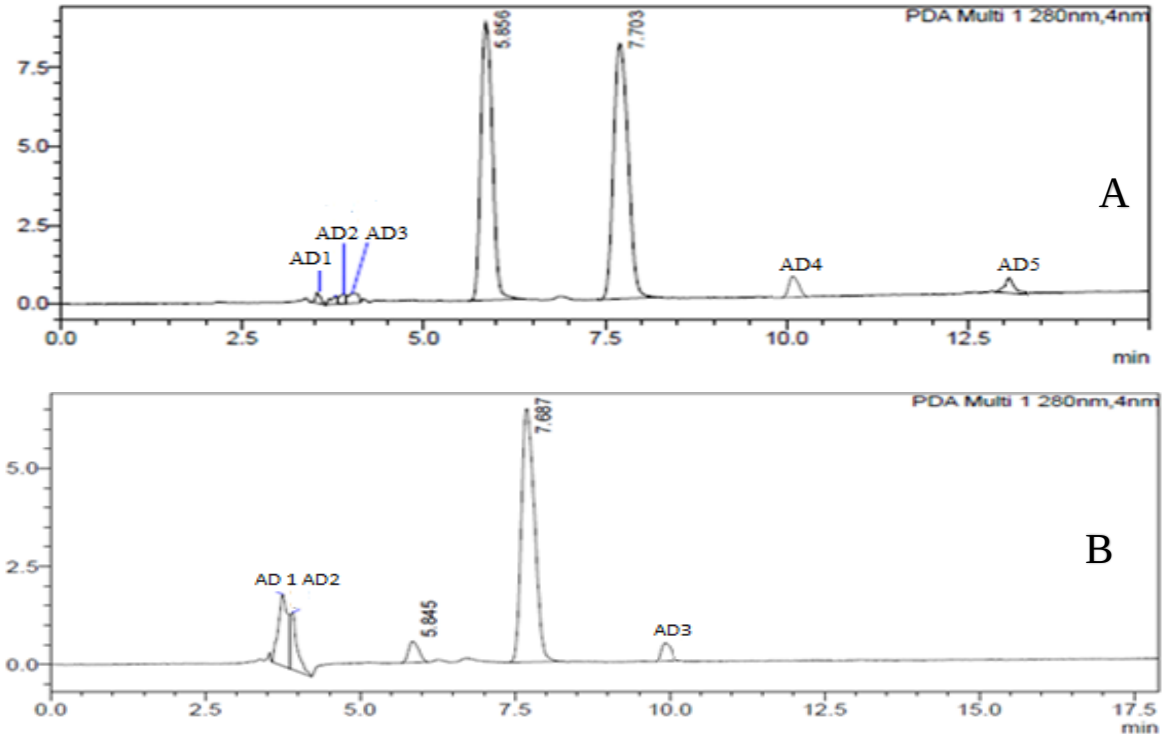


Figure 5.3.2: UFLC chromatograms of Acid Degradation A) NBL-VAL (1:1) B) NBL-VAL (1:16)

Base Degradation Studies

Base degradation studies were performed on a physical mixture of NBL-VAL in 1 N NaOH. The mixture of drugs in 1:1 and 1:16 ratio was added, separately, in 50 ml NaOH solution and kept at room temperature (27°C ± 2) with occasional stirring. Samples were withdrawn at 1 to 7 days and neutralised with HCl and diluted with diluent and filtered prior to injection. The base degradation chromatogram of NBL-VAL was depicted in figure 5.3.3. Three DPs were observed in a base degradation study (1:1).

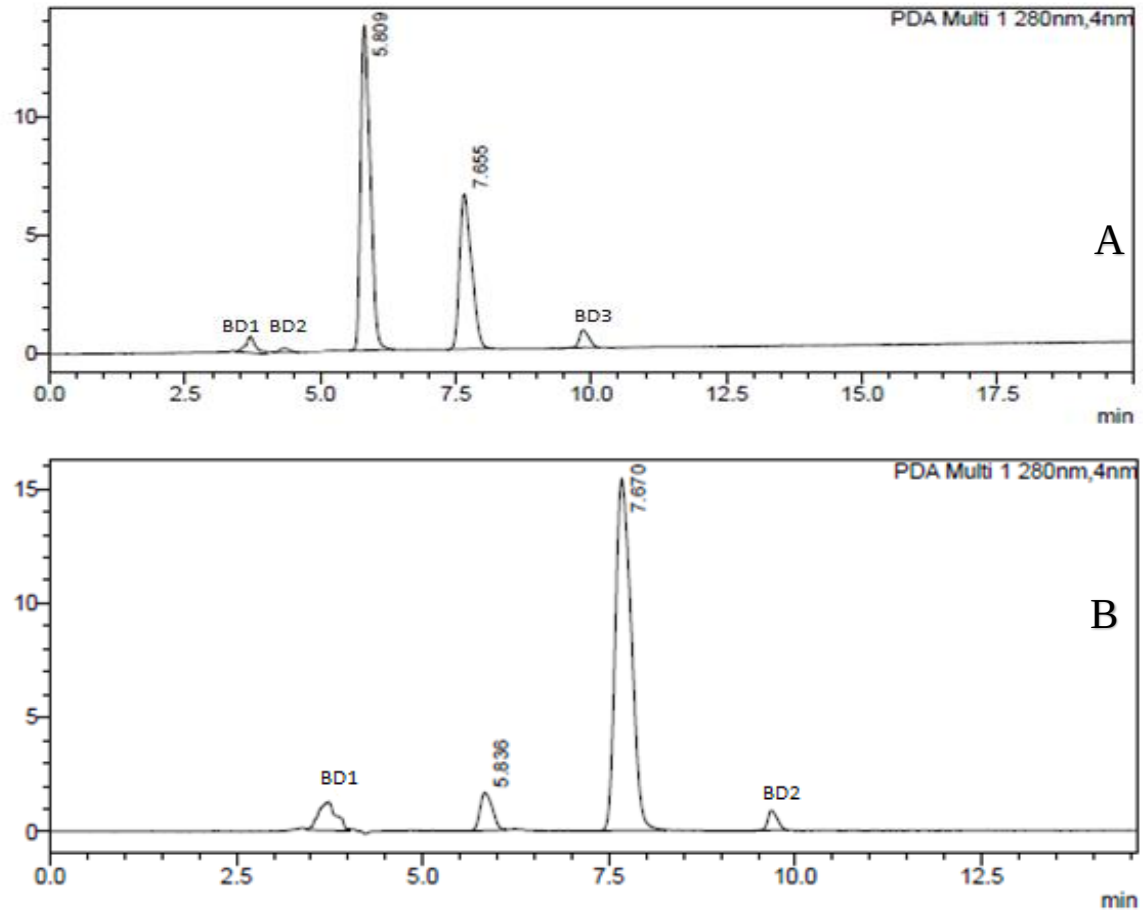


Figure 5.3.3: UFLC chromatograms of Base Degradation A) NBL-VAL (1:1)
B) NBL-VAL (1:16)

Oxidative degradation studies

Oxidative degradation studies were performed on a physical mixture of NBL-VAL in 10% H_2O_2. The mixture of drugs in 1:1 and 1:16 ratio was added, separately, in 50 ml H_2O_2 solution and kept at room temperature (27°C ± 2) with occasional stirring. Samples were withdrawn at 1 to

7 days, diluted with diluent, and filtered prior to injection. The oxidative degradation chromatogram of NBL-VAL was depicted in figure 5.3.4. One DP was observed in both studies.

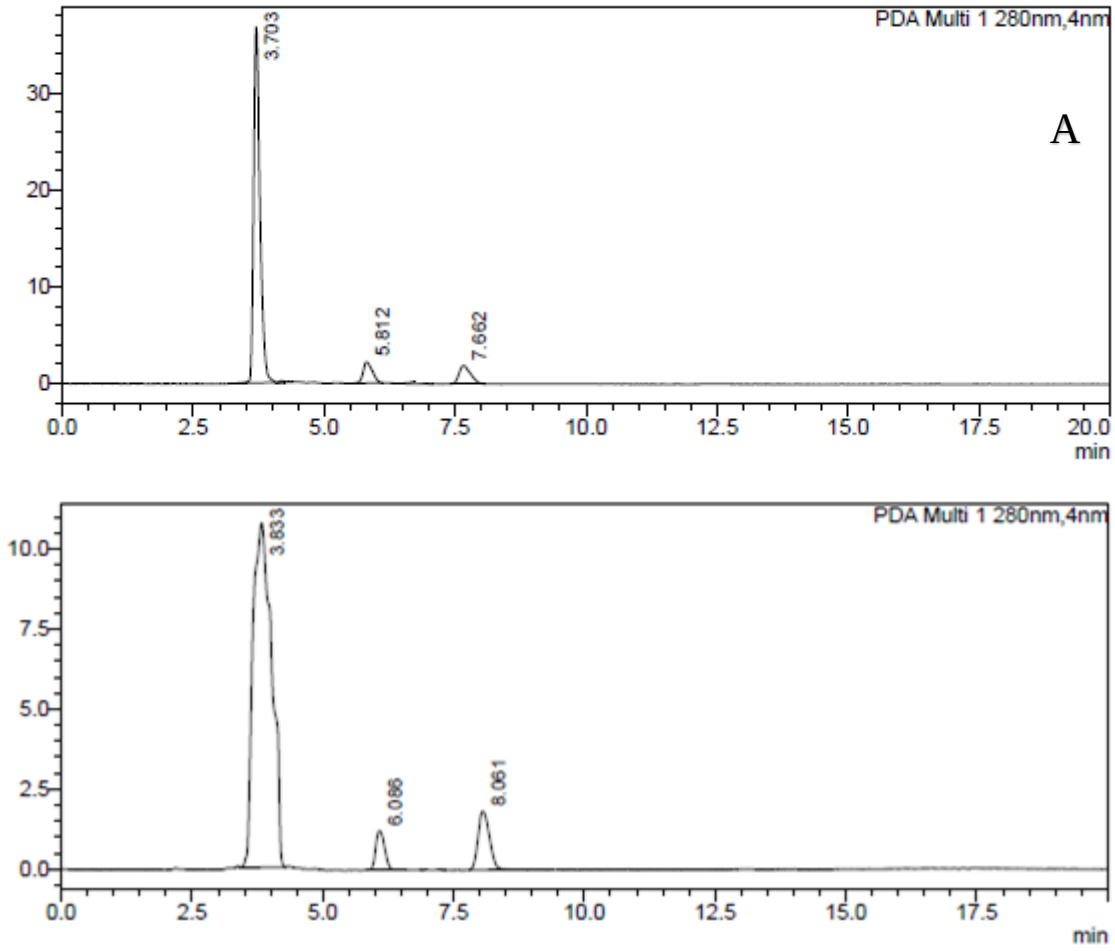


Figure 5.3.4: UFLC chromatograms of Oxidative Degradation A) NBL-VAL (1:1) B) NBL-VAL (1:16)

Thermal Degradation:

Thermal degradation studies were performed on a physical mixture of NBL-VAL. A mixture of drugs in 1:1 and 1:16 ratio was exposed to a controlled temperature oven at 80 °C ± 2 °C for 7 days. Samples were withdrawn at 1 to 7 days, diluted with diluent, and filtered prior to injection. No degradation was observed in the thermal stress study. It indicates that the NBL-VAL physical mixture was stable in the thermal stress study.

Photo-degradation Studies

Photo-degradation Studies were performed on a physical mixture of NBL-VAL. A mixture of drugs in 1:1 and 1:16 ratio was spread separately on a glass dish in a layer that was less than 2 mm thick. The drug was exposed to UV radiation in a UV light chamber. Samples were withdrawn at 1 to 7 days, diluted with diluent, and filtered prior to injection. One DP was found in a photo-degradation study.

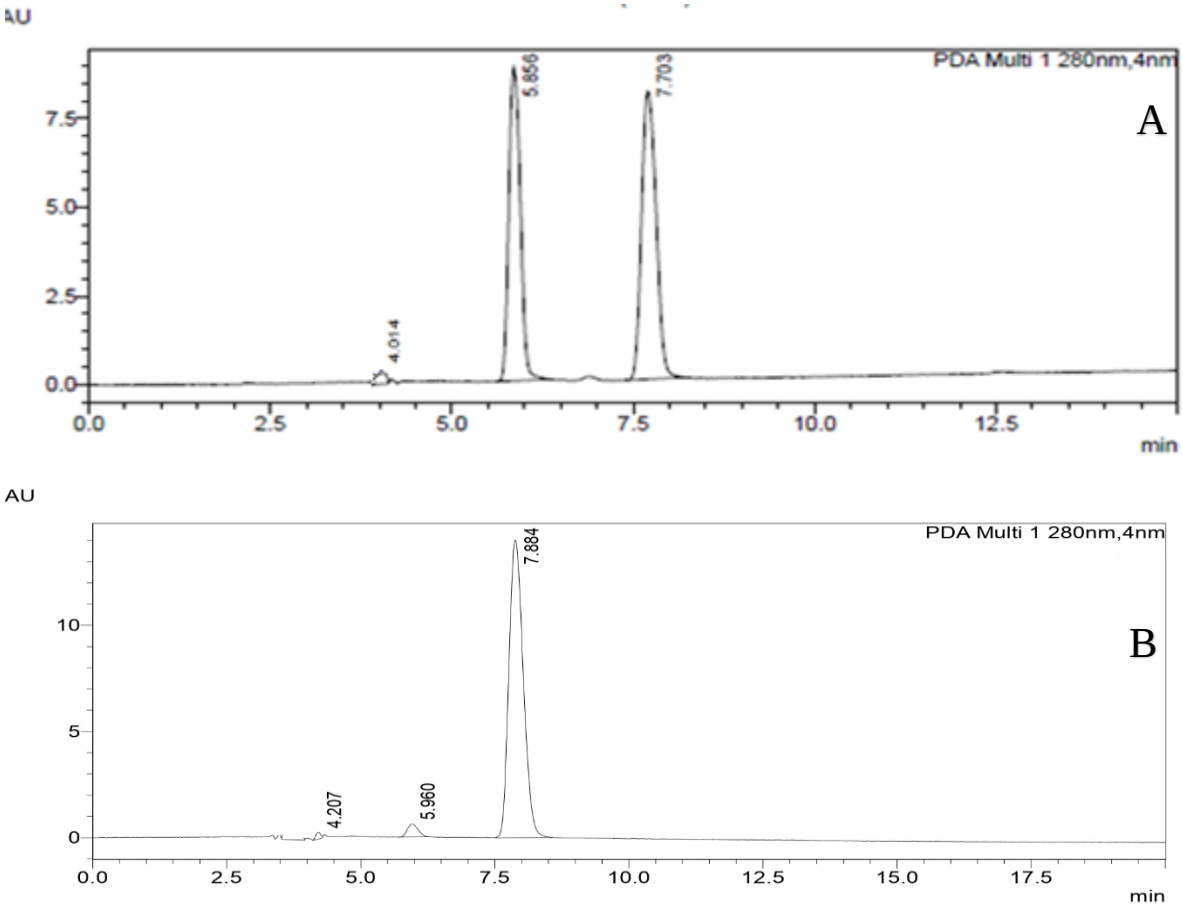


Figure 5.3.5: UFLC chromatograms of Photo-degradation A) NBL-VAL (1:1)
B) NBL-VAL (1:16)

Method Validation

Developed Stability Indicating the UFLC method was validated with the help of ICH guidelines. Validation of the optimized chromatographic method was conducted based on guidelines issued by the ICH. A set of tests was conducted to measure linearity, specificity, accuracy, LOD, LOQ, range, precision, robustness, and system suitability.

Linearity: The analytical method developed for NBL-VAL was confirmed to be linear by plotting the calibration curve between peak areas (mAU) and the concentration of the analytes (µg/mL) (Figure 2.3.5). The correlation coefficients (R2) were found to be 0.9996 and 0.9977 for NBL and VAL, respectively. The precision of the method was established through repeatability and intermediate studies/inter-day precision studies, where the % RSD of the peak areas of NBL and VAL were both well within 1%. Repeatability was confirmed by injecting six replicate injections.

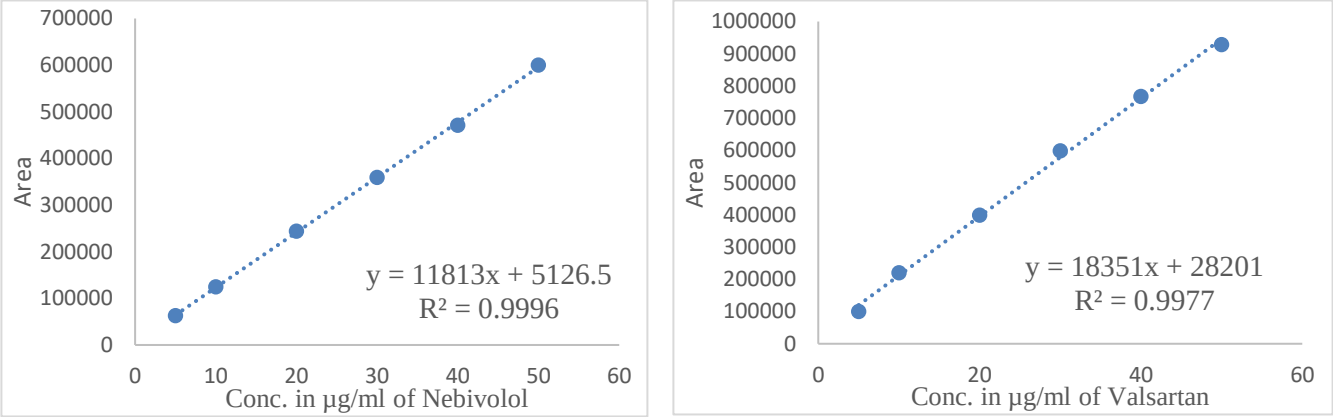


Figure 5.3.6: Linearity curve for A) NBL and B) VAL

Precision: The precision of the developed method was determined by calculating the relative standard deviation for the peak area. Using a 10 µg/mL concentration, six measurements were used to assess the repeatability. Three concentrations (5, 10, and 20 µg /mL) of intermediate precision were measured by three analysts on different days in triplicates. % RSD of NBL and VAL peak areas were well within 1% in both repeatability and inter-day precision studies.

Specificity: Specificity studies evaluate whether a method can execute analyte evaluation explicitly even when there are numerous components present including a matrix, impurities, DPs, etc. The specificity of the method was determined by chromatographic runs with standard solutions of NBL and VAL, as well as sample solutions of NBL-VAL marketed formulations at a concentration of 10 µg/mL.

Accuracy: In an analytical procedure, the accuracy is determined by how close the test result is to the accepted reference value. Three different levels of NBL and VAL concentrations were

spiked to determine the accuracy of the analytical procedure. Using the stock solution, a 10 g/mL solution of a drug was prepared and spiked at concentrations of 80%, 100%, and 120% of the theoretical NBL and VAL concentration. Triple samples of each spike level were prepared. An analysis of the standard spiked solution of NBL and VAL showed a % recovery between 99.24 - 100.42%. The developed method showed a high level of accuracy with a % RSD between 0.041 and 0.064.

LOD and LOQ: The LOD refers to the minimum detectable level of the analyte in a sample, while the LOQ represents the minimum quantifiable level with a sufficient level of accuracy and reproducibility. For NBL, the LOD and LOQ values were determined to be 0.045 µg/mL and 0.148 µg/mL, respectively, and for VAL, the corresponding values were 0.028 µg/mL and 0.092 µg/mL. These results demonstrate that the developed method has enhanced sensitivity for the reliable detection and quantification of the analytes.

Robustness: The robustness of the method was demonstrated by the insensitivity of responses towards deliberate slight variations in chromatographic parameters. During normal usage, this parameter indicates how consistently a method has been developed. Examples of variations used in this experiment were pH (± 0.1), mobile phase composition (± 5%), and flow rate (± 0.1 mL/min). CAAs such as NTP, Rt, and Tf were not significantly affected by changes in pH, flow rate, and composition of the mobile phase during different chromatographic conditions. Consequently, the insensitivity of the method's responses to small changes in conditions reveals the robustness of the developed UFLC method.

System suitability testing: For the system suitability test, a standard solution of 10 µg/mL was injected six times in triplicate into the chromatographic system. Prior to sample analysis, the %.RSD of peak area and Rt were determined, which were found to be acceptable below 1%. It is recommended to maintain the criteria of accepting theoretical plates greater than 2000 and Tf less than 2.0. There were no striking differences in peak area, NTP, Rt, and Tf of NBL-VAL between six replicates in the system suitability testing. The developed analytical method proved to be highly accurate as the %RSD for studied parameters was below 1%.

Solution Stability: The stability of the NBL-VAL solution was established experimentally over 10 days in a volumetric flask stored at room temperature (27 ± 2 °C). The % assay was found to be 99.35% and 99.72% for NBL and VAL, respectively, which is within the limit of acceptance.

Results

Several preliminary studies were carried out to develop a simple, robust, and stability-indicating UFLC method for evaluating NBL-VAL from bulk and formulation samples. Rapid chromatographic separations were obtained with lower Rt values, and peak symmetry was acceptable with a flow rate of 0.8 mL/min, and column temperature of 25-27 ± 2°C. Optimal chromatographic separation was attained by ACN: MeOH: Ammonium acetate buffer (20 mM, pH 3.75 adjusted by formic acid) in a 30: 40: 30 ratio (% v/v/v) as the mobile phase.

To achieve the desired separation of the drug sample and formed DPs, various experiments were conducted on a Kromasil C18 column. The UFLC studies were initially conducted on an individual sample under each stress condition, and later on a mixture of stressed samples. The degradants formed during the stability study were successively separated by employing the developed UFLC method. Furthermore, ICH Guidelines Q2(R1) were followed for the validation of the developed stability indicating the UFLC method.

Assay of NBL-VAL

In order to establish the applicability of the developed UFLC method to commercial formulations, the validated method was applied for the analysis of NBL-VAL formulation. The average results of the assay were found to be 99.18 and 99.84 ± 0.02, respectively. Subsequently, this method can be applied for routine analysis of NBL-VAL due to its short analysis time and low RSD values.

Discussion

Stability Behaviour of Physical Mixture of NBL-VAL

A forced degradation study was performed on a physical mixture of NBL-VAL, to evaluate the stability behaviour of these two drugs. In the acid stress study, five DPs (AD_1-AD_5) were

observed in a 1:1 ratio while three were in a 1:16 ratio. The absence of the other DP on the chromatogram could be attributed to the variations in area and height between the drug and the other DPs. Three DPs were detected in the base degradation study (BD_1-BD_3) in a 1:1 ratio, while two were in a 1:16 ratio. In the photo and oxidative degradation study, one degradation product was observed. No degradant was observed in the thermal degradation study for the NBL-VAL physical mixture. Table 5.3.2 contains the summary of the forced degradation study for NBL-VAL.

The stability study samples were analysed using LC-MS/MS to determine the structure of the degradants. The fragmentation pattern and mass spectra were examined to identify the most suitable structure for each degradant. Furthermore, an *in silico* toxicity assessment study was conducted for each structure of the degradants.

Table 5.3.2 Summary of forced degradation study for NBL-VAL

Stress condition	No. of degradants observed	Rt of degradants
Acid degradation	5	AD_1 - 3.717, AD_2 - 3.868, AD_3 - 4.014, AD_4 - 10.194, AD_5 - 13.124
Base degradation	3	BD_1 - 3.703, BD_2 - 4.298, BD_3 - 9.948.
Oxidative degradation	1	OD_1 - 3.703
Photo-degradation	1	PD_1 - 4.014
Thermal degradation	0	-

STABILITY INDICATING UFLC METHOD DEVELOPMENT AND VALIDATION FOR SACUBITRIL-VALSARTAN

Standard Solution Preparation

A precisely weighed 10 mg drug (SAC-VAL) was transferred into a clean 100 ml volumetric flask. The drug samples were dissolved in separate volumetric flasks in 5 ml water by vigorous shaking and ultrasonication. A standard solution of 100 µg/ml was prepared using the mobile phase as diluent. Using serial dilution, working standard solutions were prepared to achieve an appropriate concentration (5-50 µg/ml).

Sample Solution Preparation

A clean and dry mortar was used to crush, an accurately weighed twenty tablets of SAC-VAL (Cidmus 50). The powder equivalent to the label claim was (SAC-VAL 24/26 mg) transferred into a 100 ml volumetric flask. The powder was dissolved in 10 ml water: MeOH (1:1) solution with ultrasonication and a mechanical shaker. Using serial dilution, a working sample solution was prepared by centrifuging the resultant stock solution for 10 minutes at 5000 rpm, in order to achieve a final concentration of 24/26 ug/ml of SAC-VAL, respectively. Prior to analysis, all the standard and sample solutions were filtered through 0.22µ membrane filters.

UFLC Method Development

The chromatographic separation was attained by using Shimadzu UFLC, with Kromasil C18 column (250 × 4.6 mm i.d., particle size 5 µm), as the stationary phase, and MeOH: ACN: Ammonium acetate buffer (25 mM, pH adjusted to 4 using formic acid) (40:30:30 v/v/v) as mobile phase. The QbD-enabled UFLC method was developed using isocratic mode with a 0.8 ml/min flow rate throughout the chromatographic run. The injection volume was 10 ul. The eluent was monitored by a PDA detector for the quantitation of SAC-VAL and its DPs. All determinations were performed at room temperature. The following table contains the chromatographic conditions for the physical mixture of SAC-VAL.

Table 5.4.1: Optimised chromatographic conditions of developed UFLC method for SAC-VAL

Sr. No.	Parameters	Description/ Value
1.	Stationary Phase	Thermo C18 column- 250 × 4.6 mm with a 5 µm particle size.
2.	Mobile Phase	MeOH: ACN: Ammonium acetate buffer (25 mM, pH adjusted to 4 using formic acid) (40:30:30 v/v/v)
3.	Flow Rate	0.8 ml/min
4.	Detector	SPD-M20A Prominence diode array detector.
5.	Elution	Isocratic
6.	Injection Volume	10 µl
7.	Column temperature	Room temperature (25 ± 2 °C)
8.	Run time	20 min

9.	Diluent	Mobile Phase.	
10	Retention Time*	SAC – 5.200	VAL- 7.756
11	Tailing factor*	SAC – 1.106	VAL- 1.117
12	NTP (USP)*	SAC - 5913	VAL- 7894
13	Resolution (USP)	5.324	

*= value represents the average of six determinates (n=6)

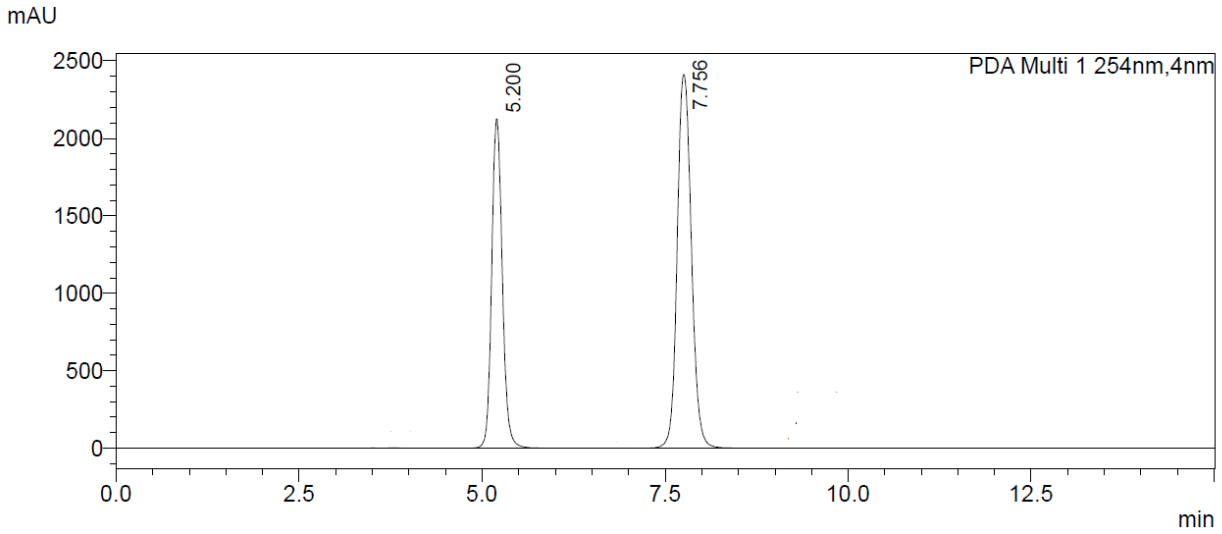


Figure 5.4.1: UFLC Chromatogram for SAC-VAL physical mixture

Force Degradation Studies on Physical Mixture of SAC-VAL

ICH guidelines Q1A(R2) were followed for the stress tests. The pure drug and physical mixture were exposed to various stress conditions like hydrolysis, photolysis, thermal, and oxidation. The stock solution of pure drug and the physical mixture of the drug was prepared in MeOH. Force Degradation Studies were performed on a physical mixture of SAC-VAL, to assess the stability behaviour of both drugs.

Stress Sample Preparation for UFLC Analysis

The stress samples were diluted with diluent up to 10 ml after exposure to acidic, basic, oxidative, and solution-state photo-degradation conditions. The solid-state samples of thermal and photo stress were weighed suitably and diluted with diluent. Using a 0.22µ membrane filter, all

the prepared stressed samples were filtered and used to develop a stability indicating UFLC method.

Acid Degradation Studies

Acid degradation studies were performed on a physical mixture of SAC-VAL in 1 N HCl. A 50 mg mixture of drugs (1:1) was added, in 50 ml 1 N HCl solution and kept at room temperature (27°C ± 2) with occasional stirring. Samples were withdrawn at 1 to 7 days and neutralised with 1 N NaOH solution and diluted with diluent and filtered prior to injection. The acid degradation chromatogram for the SAC-VAL physical mixture was shown in figure 5.4.2. Overall, seven DPs were observed in Acid degradation studies. (AD1 – 3.362, AD2 – 3.624, AD3 – 6.014, AD4 – 7.248, AD5 – 9.118, AD6 – 10.308, AD7 – 13.495)

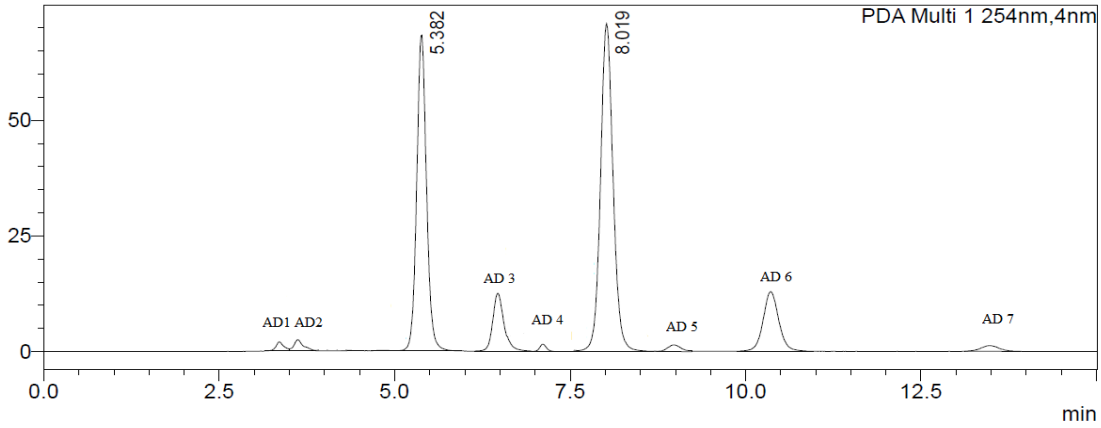


Figure 5.4.2: Acid degradation chromatogram for SAC-VAL physical mixture

Base Degradation Studies

Base degradation studies were performed on a physical mixture of SAC-VAL in 1 N NaOH. A 50 mg mixture of drugs (1:1) was added, in 50 ml 1 N NaOH solution and kept at room temperature (27°C ± 2) with occasional stirring. Samples were withdrawn at 1 to 7 days and neutralised with 1 N HCl solution and diluted with diluent and filtered prior to injection. The base degradation chromatogram for the SAC-VAL physical mixture was depicted in figure 5.4.3. Overall, six DPs were observed in Base degradation studies. (BD1 – 3.384, BD2 – 3.632, BD3 – 6.376, BD4 - 7.248, BD5 – 10.198, BD6 – 13.182)

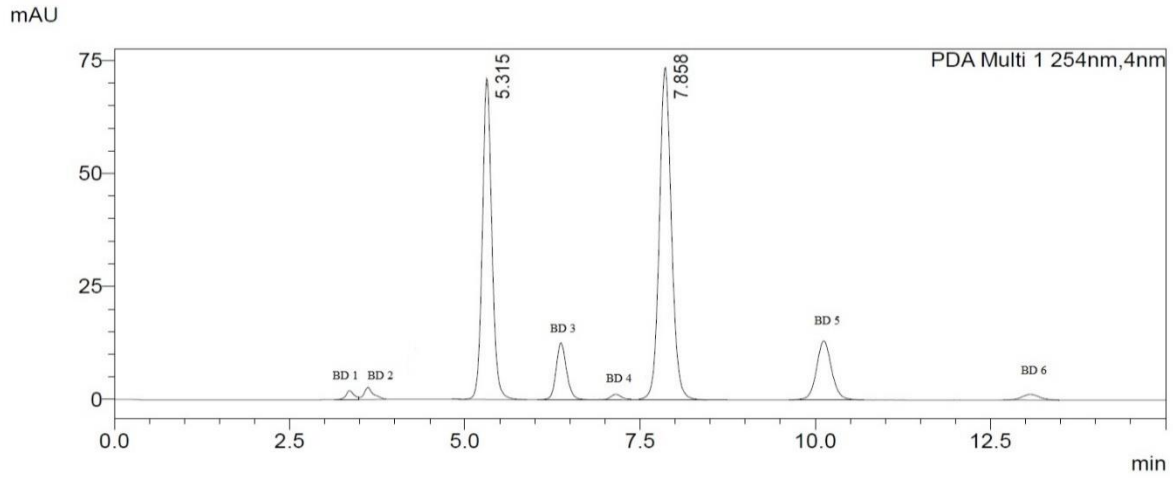


Figure 5.4.3: Base degradation chromatogram for SAC-VAL physical mixture

Oxidative degradation studies

Oxidative degradation studies were performed on a physical mixture of SAC-VAL in 10% H_2O_2. A 50 mg mixture of drugs (1:1) was added, in 50 ml H_2O_2 solution and kept at room temperature (27°C ± 2) with occasional stirring. Samples were withdrawn at 1 to 7 days, diluted with diluent, and filtered prior to injection. The oxidative degradation chromatogram for the SAC-VAL physical mixture was illustrated in figure 5.4.4. Overall, three DPs were observed in the Oxidative degradation study. (OD1 – 3.012, OD2 – 4.207, OD3 – 6.212)

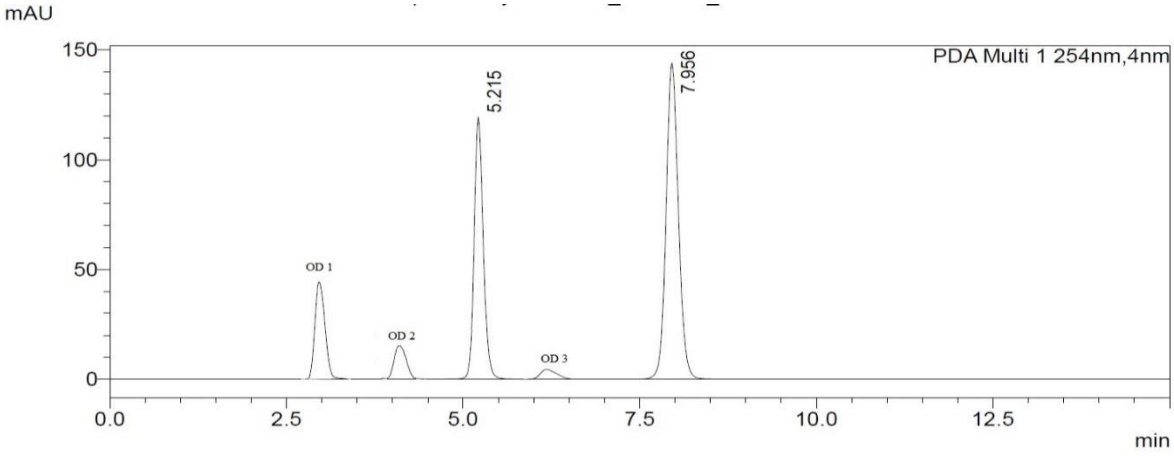


Figure 5.4.4: Oxidative degradation chromatogram for SAC-VAL physical mixture

Photo-degradation Studies

Photo-degradation Studies were performed on a physical mixture of SAC-VAL. Approximately 100 mg mixture of SAC-VAL (1:1) was spread on a glass dish in a layer that was

less than 2 mm thick. The drug was exposed to UV radiation in a UV light chamber. Samples were withdrawn at 1 to 7 days, diluted with diluent, and filtered prior to injection. The thermal degradation chromatogram for SAC-VAL Physical mixture was illustrated in figure 5.4.5. Two DPs were observed in the photo-degradation study. (PD1 – 2.902, PD2 – 3.981)

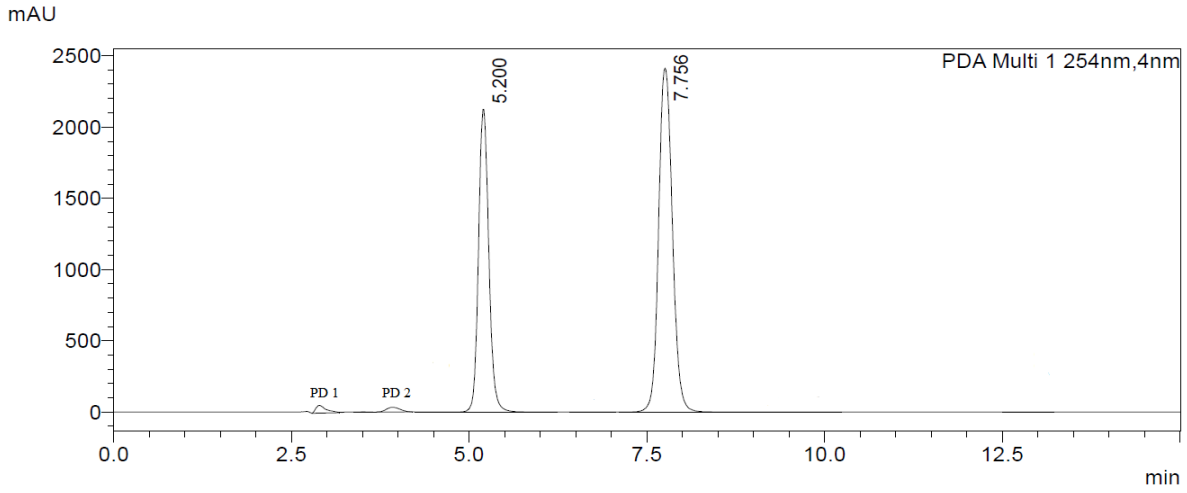


Figure 5.4.5: Photo-degradation chromatogram for SAC-VAL physical mixture

Thermal Degradation

Thermal Degradation Studies were performed on a physical mixture of SAC-VAL. Approximately 100 mg mixture of SAC-VAL (1:1) was spread on a glass dish in a layer that was less than 2 mm thick and exposed to a controlled temperature oven at 80 °C ± 2 °C for 7 days. Samples were withdrawn at 1 to 7 days, diluted with diluent, and filtered prior to injection. The photo-degradation chromatogram for SAC-VAL Physical mixture was shown in figure 5.4.6. One DP was observed in thermal degradation. (TD1 – 4.359)

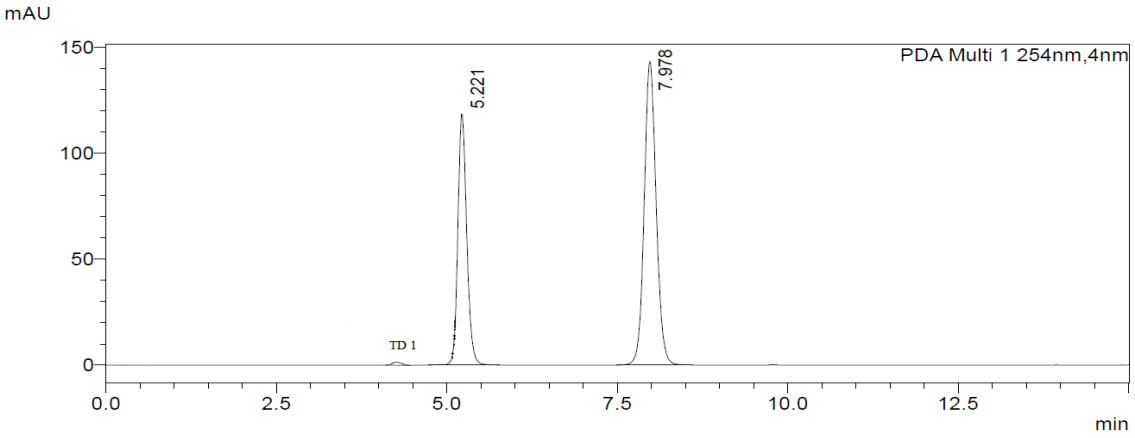


Figure 5.4.6: Thermal degradation chromatogram for SAC-VAL physical mixture

Method Validation

Validation of the optimized chromatographic method was conducted based on guidelines issued by the ICH. A set of tests was conducted to measure linearity, specificity, accuracy, LOD, LOQ, range, precision, robustness, and system suitability.

Linearity. In a linear analytical method, the concentration of an analyte in the sample is directly proportional to the test results. A concentration range of between 5 and 50 µg/mL was used to serially dilute the standard stock solution for estimation. Furthermore, to generate the calibration curve, the peak area was plotted against drug concentration. For each concentration, triplicate analyses (n=3) were conducted. The method was found to be linear for SAC-VAL, with a correlation coefficient (R^2) of 0.9997 and 0.9984, respectively.

Precision: Repeated measurements of homogenous samples were used to determine the precision of an analytical method. Moreover, the precision of the developed method was determined by calculating the relative standard deviation for the peak area. Using a 10 µg/mL concentration, six measurements were used to assess the repeatability. Three concentrations (5, 10, and 20 µg /mL) of intermediate precision were measured by three analysts on different days in triplicates. % RSD of SAC and VAL peak areas were well within 1% in both repeatability and inter-day precision studies.

Specificity. Specificity studies evaluate whether a method can execute analyte evaluation explicitly even when there are numerous components present including a matrix, impurities, degradation products, etc. The specificity of the method was determined by chromatographic runs with standard solutions of SAC and VAL, as well as sample solutions of SAC-VAL marketed formulations at a concentration of 10 µg/mL.

Accuracy. In an analytical procedure, the accuracy is determined by how close the test result is to the accepted reference value. Three different levels of SAC and VAL concentrations were spiked to determine the accuracy of the analytical procedure. Using the stock solution, a 10 g/mL solution of a drug was prepared and spiked at concentrations of 80%, 100%, and 120% of the theoretical SAC and VAL concentration. Triple samples of each spike level were prepared. An analysis of the

standard spiked solution of SAC and VAL showed a % recovery between 99.62 % and 100.38%. With % RSD between 0.038 and 0.044, the developed method had an extremely high degree of accuracy.

LOD and LOQ: LOD represents the lowest amount of analyte in a sample that can be detected but not necessarily quantified as an exact value. LOQ represents the lowest amount of analyte in the sample that can be quantified with an acceptable level of repeatability and accuracy. The LOD values for VAL and SAC were 0.0042 µg/mL and 0.0046 µg/mL, respectively. The LOQ values for the VAL and SAC were 0.0138 µg/mL and 0.0151 µg/mL, respectively. These results led to the conclusion that the developed method has improved sensitivity for its effective detection and quantification.

Robustness: An analytical technique's robustness refers to its ability to withstand small, but deliberate, changes in parameters. During normal usage, this parameter indicates how consistently a method has been developed. Examples of variations used in this experiment were pH (± 0.25), mobile phase composition (± 5%), and flow rate (± 0.1 mL/min). CAAs such as NTP, Rt, and Tf were not significantly affected by changes in pH, flow rate, and composition of the mobile phase during different chromatographic conditions. Consequently, the insensitivity of the method's responses to small changes in conditions reveals the robustness of the developed UFLC method.

System Suitability Testing: For the system suitability test, a standard solution of 10 µg/mL was injected six times in triplicate into the chromatographic system. Prior to sample analysis, the %.RSD of peak area and Rt were determined, which were found to be acceptable below 2%. It is recommended to maintain the criteria of accepting theoretical plates greater than 2000 and Tf less than 2.0. There were no striking differences in peak area, NTP, Rt, and Tf of SAC-VAL between six replicates in the system suitability testing. The developed analytical method proved to be highly accurate as the %RSD for studied parameters was below 1%.

Solution Stability: The stability of the SAC-VAL solution was established experimentally over 10 days in a volumetric flask stored at room temperature (30 ± 2 °C). The % assay was found to be 99.46% and 99.54% for SAC and VAL, respectively, which is within the limit of acceptance.

Results

Several preliminary studies were carried out in order to develop a simple, robust, and stability-indicating UFLC method for evaluating SAC-VAL from bulk or formulation samples. Rapid chromatographic separations are obtained with a lower Rt value, and peak symmetry was acceptable with a flow rate of 0.8 mL/min, column temperature of 27 ± 2 °C, and UV detection at 254 nm.

A cost-effective and straightforward liquid chromatographic method was developed by using MeOH, CAN, and Ammonium acetate buffer (25 mM, pH 4 adjusted with formic acid) (40: 30: 30 v/v/v) as mobile phase. ICH Guideline Q2(R1) was followed for the validation of the developed stability indicating the UFLC method.

Assay of SAC-VAL formulation

In order to establish the applicability of the developed UFLC method to commercial formulations, the validated method was applied for the analysis of SAC-VAL formulation. The average results of the assay were found to be 99.38 ± 0.02 and 99.54 ± 0.02, respectively. Subsequently, this method can be applied for routine analysis of SAC-VAL due to its short analysis time and low RSD values.

Discussion

Stability Behaviour of Physical Mixture of SAC-VAL

A forced degradation study was performed on a physical mixture of SAC and VAL, to evaluate the stability behaviour of these drugs. To confirm the specificity and capability of the developed method, forced degradation studies were conducted according to the ICH recommendations. In the acid stress study, a total of seven degradation products (AD_1-AD_7) were observed and in the base degradation study, a total of six degradation products (BD_1-BD_6) were observed. In the degradation study involving acid and base conditions, it was observed that six out of the seven degradants had nearly similar Rts. In the oxidative degradation study, three degradation products (OD_1-OD_3) were observed. In the thermal degradation study, one degradation product (TD_1) was observed and in the photo-degradation study, two degradation products (PD_1-PD_2) were observed. Table 5.4.2 provide the summary of the forced degradation of SAC-VAL.

Table 5.4.2 Summary of forced degradation study for SAC-VAL

Stress condition	No. of degradants observed	Rt of degradants
Acid degradation	7	AD1 – 3.362, AD2 – 3.624, AD3 – 6.014, AD4 – 7.248, AD5 – 9.118, AD6 – 10.308, AD7 – 13.495
Base degradation	6	BD1 – 3.384, BD2 – 3.632, BD3 – 6.376, BD4 - 7.248, BD5 – 10.198, BD6 – 13.182
Oxidative degradation	3	OD1 – 3.012, OD2 – 4.207, OD3 – 6.212
Photo-degradation	2	PD1 – 2.902, PD2 – 3.981
Thermal degradation	1	TD1 – 4.359

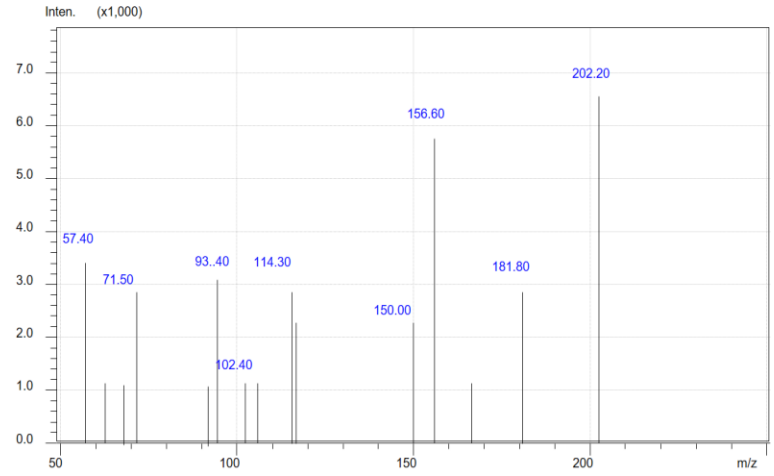
LC-MS/MS CHARACTERISATION OF FORMED DEGRADANTS

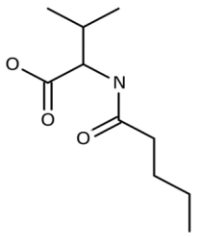
The degradants sample were analysed with the help of the developed UFLC method and the characterisation of formed degradants was performed with the help of LC-MS/MS. The instrument parameters were optimized appropriately for LC-MS/MS studies, in order to accurately determine the precise masses of both the molecular ions and the DP fragments. The details of optimised LC-MS/MS instrument parameters were given in table 5.5.1.

Table 5.5.1: LC-MS/MS optimised instrumental parameters

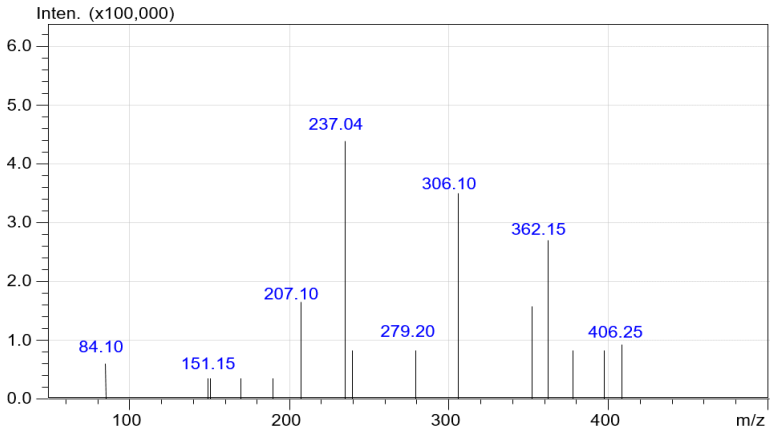
LC-MS/MS	Shimadzu LC-MS 8040, Triple Quadrupole
Mass range	2 to 2000 m/z
Ionisation source	Electrospray Ionisation (ESI)
Nebulizing gas flow	3 L/min
DL Temperature	250 ℃
Heat Block Temperature	400 ℃
Drying Gas Flow	15 L/min
Dwell Time	100 msec
LC system	Shimadzu, UFLC
Detector	PDA
Run time	15 min
Pump flow rate	0.8 ml/min

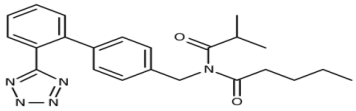
The stress sample was filtered prior to injection and the mass spectra of each degradant were studied. Zeneth prediction, drug degradation pathway, and the fragmentation patterns of the molecule were taken into consideration for the interpretation of mass spectra. The mass spectra of degradants and the most probable structure of each degradant were represented in the figure. 5.5.1. The toxicity prediction of each degradant is given in table 5.5.3.



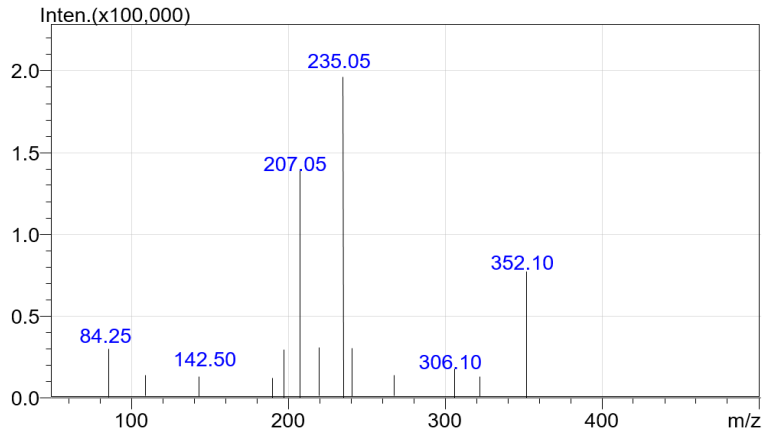


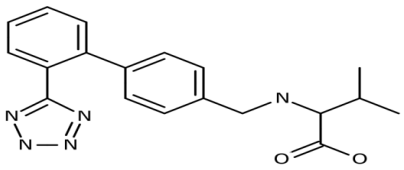
VAL DP 1





VAL DP 2





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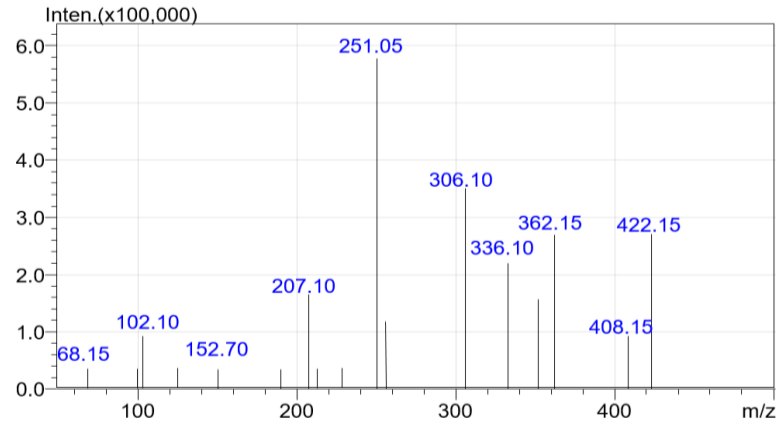
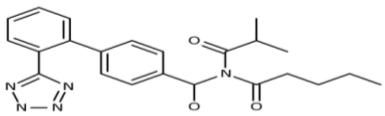
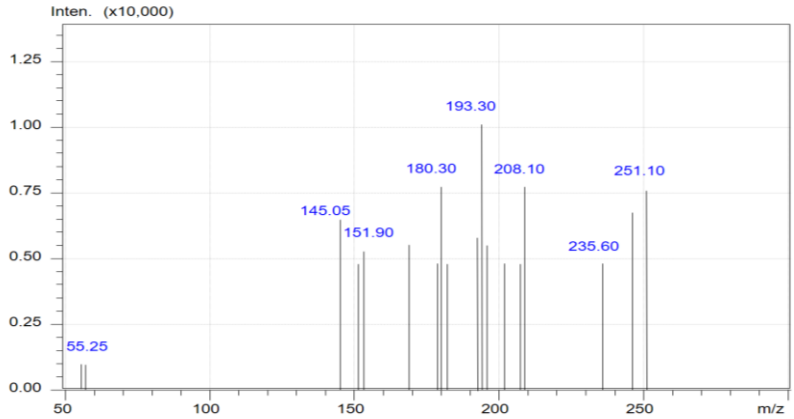
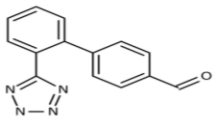


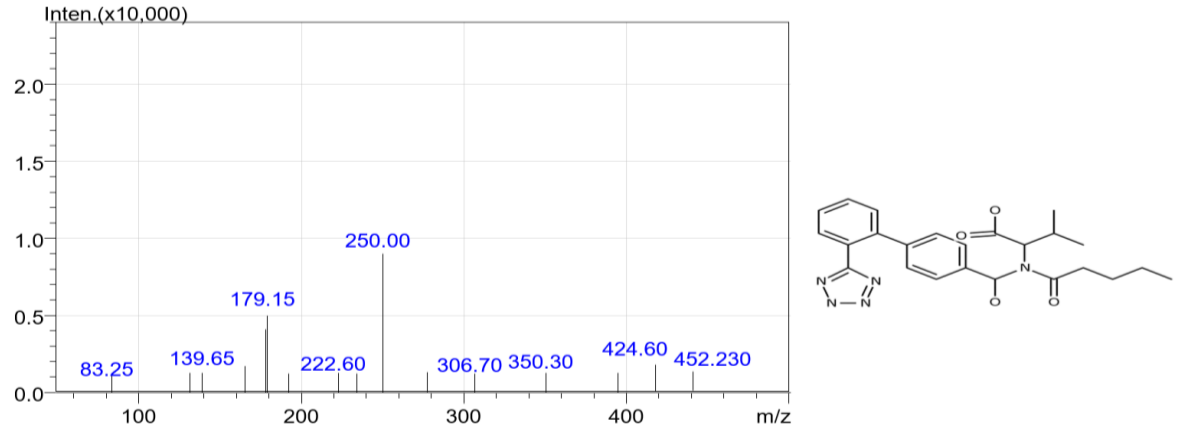


VAL DP 4

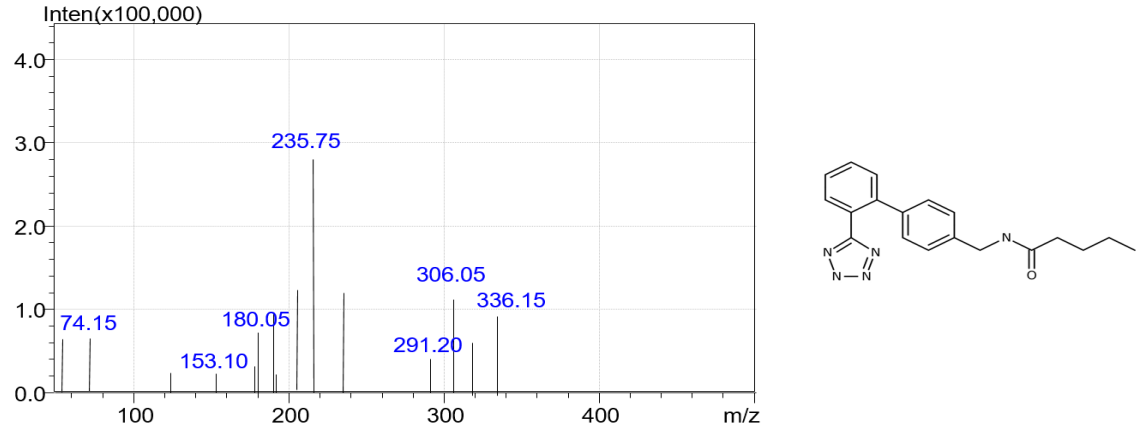




VAL DP 5



VAL DP 6



VAL DP 7

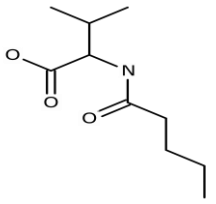
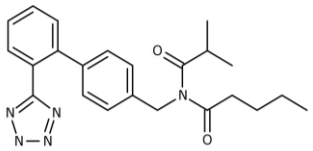
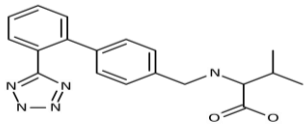
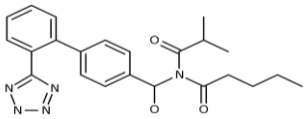
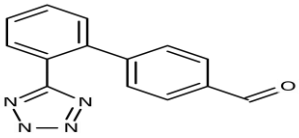
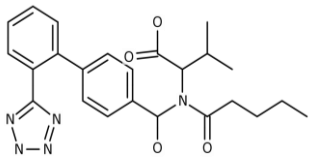
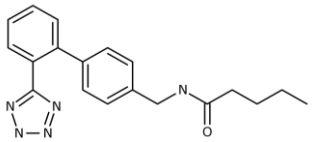
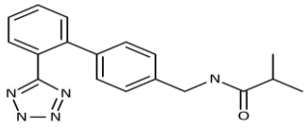
Figure 5.5.1 Mass spectra of Degradants

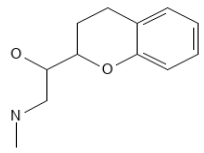
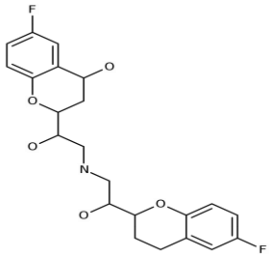
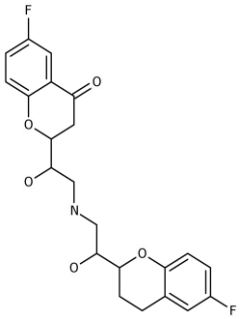
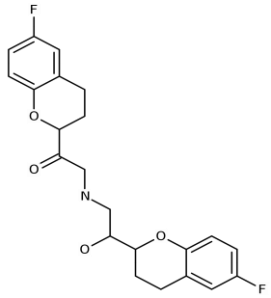
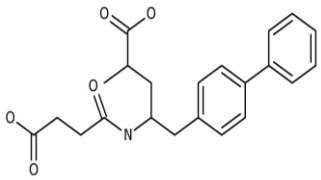
***In-silico* Toxicity Assessment of Characterized Degradants**

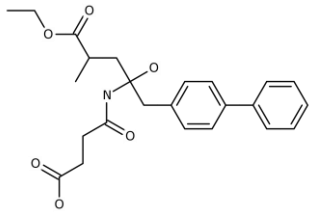
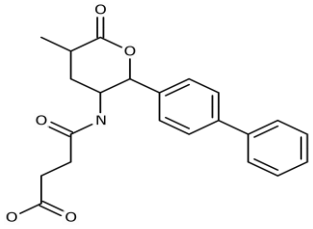
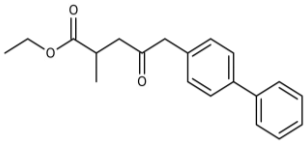
The most probable structure of degradants was predicted with the help of mass fragmentation patterns and Zeneth. Furthermore, with the help of TEST software the *in-silico* Toxicity of these characterized degradants was predicted. The details of the *in-silico* toxicity assessment of DPs were given below.

Table 5.5.3: *In-silico* toxicity assessment of characterized degradants

Name of DP	Structure	Observed for	TEST Prediction	Zeneth Score

VAL DP1		AD_1, BD_1	Non Mutagenic (0.30)	900
VAL DP2		AD_2, BD_2	Non Mutagenic (0.35)	925
VAL DP3		AD_5, BD_5	Non Mutagenic (0.35)	700
VAL DP4		AD_4, BD_4	Mutagenic 0.61 (TD2)	925
VAL DP5		OD_1	Non Mutagenic (0.30)	900
VAL DP6		AD_3, BD_3	Mutagenic (0.56) (TD1)	940
VAL DP7		OD_2	Non Mutagenic (0.30)	900
VAL DP8		PD_1	Non Mutagenic (0.32)	700

NBL DP1		AD_1	Non mutagenic (0.30)	--
NBL DP2		AD_2	Non Mutagenic (0.32)	814
NBL DP3		BD_1	Mutagenic (0.54) TD 3	814
NBL DP4		AD_3, BD_2	Non Mutagenic (0.32)	755
SAC DP1		AD_4	Non mutagenic (0.35)	700

SAC DP2		$AD_{5,}$ BD_4	Non mutagenic (0.30)	883
SAC DP3		PD_2	Non mutagenic (0.32)	700
SAC DP4		TD_1	Non mutagenic (0.35)	883

Isolation of Predicted Toxic Degradants (Genotoxic degradants)

The samples were prepared to get the maximum amount of degradant. Three predicted toxic degradants (TD1, TD2, TD3) were isolated with the help of Preparative LC. The chromatographic parameters of the developed UFLC method were used for preparative liquid chromatography for the isolation of degradants. The concentrated samples of degradant were collected. Confirmed with LC run and MS- spectra and fragmentation pattern. The resultant sample was used for genotoxicity assessment.

Genotoxicity study of predicted toxic impurities

The Ames test is an extensively used method to test the genotoxicity of chemical agents. The test uses bacterial strains that have mutations in their histidine biosynthesis pathway, making them unable to cultivate on a histidine-lacking medium. If a chemical agent causes a mutation in the bacterial DNA that reverses this mutation, the bacteria can grow on the histidine-deficient medium, indicating that the chemical agent is potentially genotoxic. The Ames test is a highly

sensitive tool used for screening potential genotoxic carcinogens. It is also known as the bacterial reverse mutation test.

AMES test performed as per ICH S2(R1) and OECD guidelines. (Test guideline no. 471 – Bacterial Reverse Mutation Test). Test performed by using *Salmonella typhimurium* TA98 bacterial strain. The agar plates were prepared and the test solution was added separately and incubated for 48 hours. The incubated plates has been depicted in figure 5.5.2. The colony counter was employed for the measurement of bacterial colonies in the incubated plates. The colony count was performed and compared between the control group and each treatment group to assess their effects. The results indicated that the number of colonies on the TD2 plate exceeded those on the other plates, suggesting that TD2 may possess genotoxic properties.

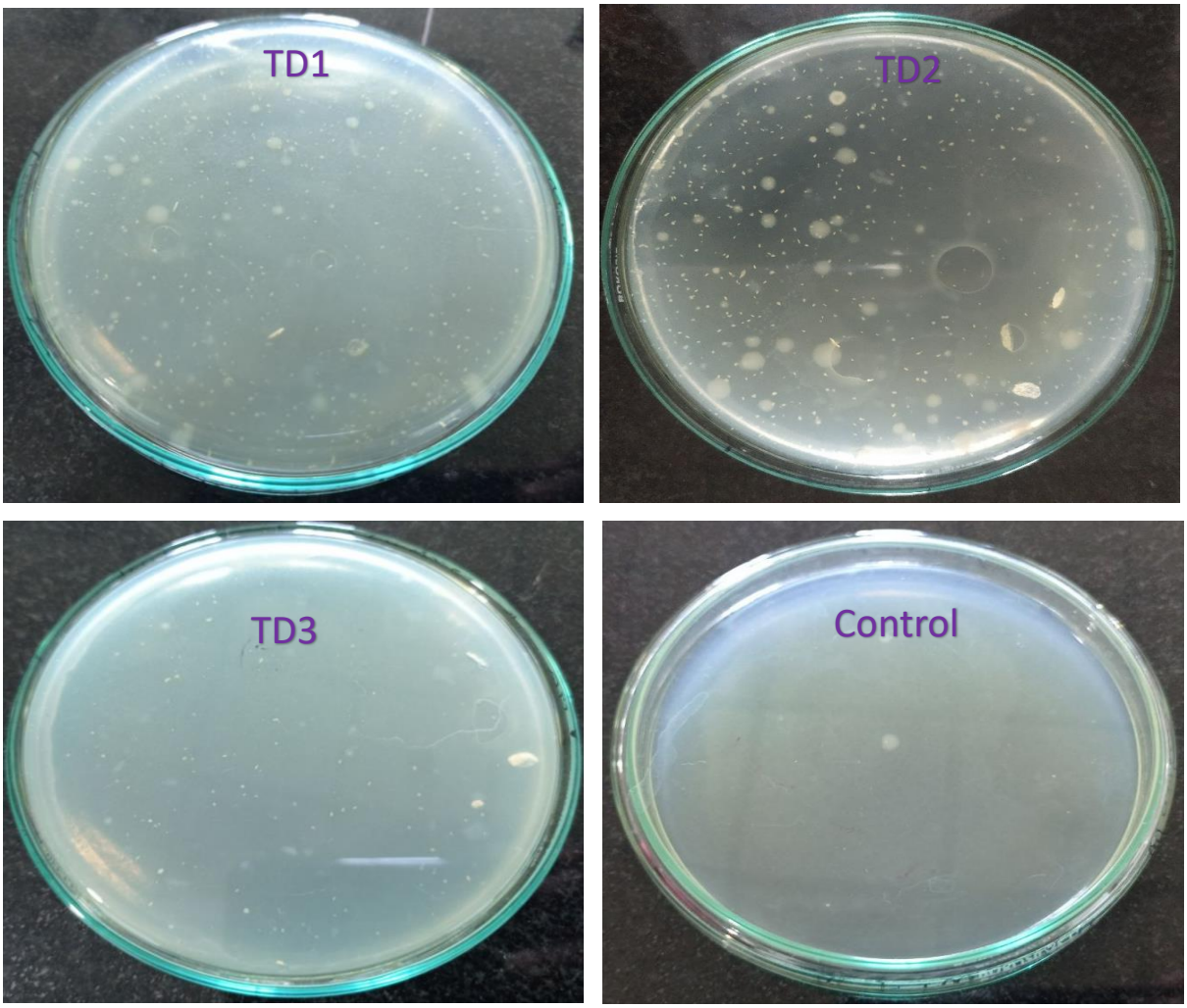


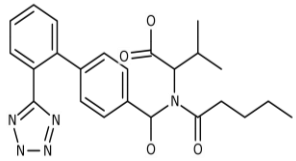
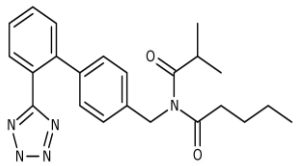
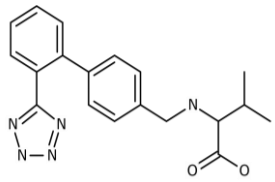
Figure 5.5.2 AMES Test – Mutagenicity confirmation of predicted toxic degradants.

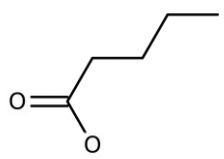
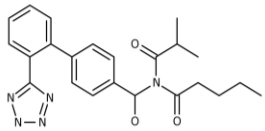
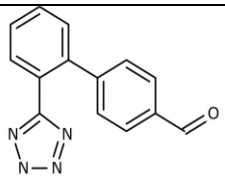
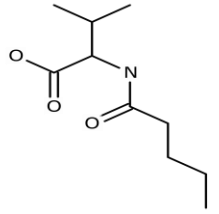
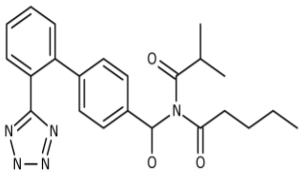
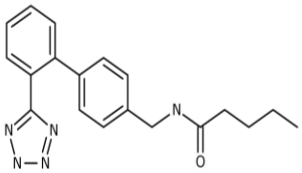
RESULTS AND DISCUSSION

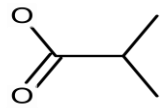
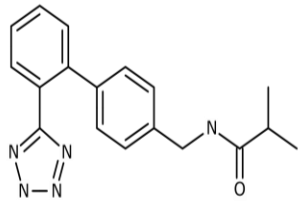
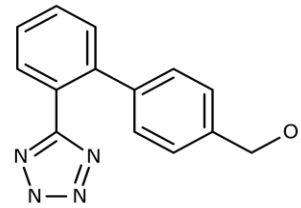
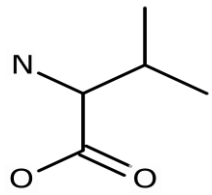
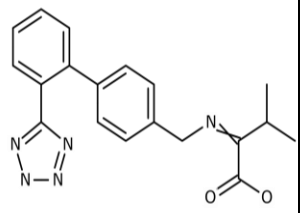
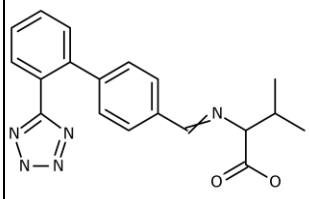
Zeneth assisted drug degradation prediction:

The degradation prediction study for each applied set of conditions was performed with the help of Zeneth software. The degradation prediction study was performed for each matrix and also in the presence of genotoxic impurities. The Zeneth predicted drug degradation results of the query compound were summarised in the following tables.

Table 6.1: Zeneth-assisted drug degradation prediction for VAL and *in-silico* toxicity assessment summary

Structure	Name	Parent	Score	Transformation Name	Exact Mass	Toxicity assessment by TEST
	D1	Q	940	Oxidation of N-alkyl amide to hemiamidal	451.222	Mutagenic (0.56) Hepatotoxic (0.52)
	D2	Q	925	Oxidative cleavage of alpha-functionalised carboxylic acid or amide	405.2165	Non-Mutagenic (0.35)
	D3	Q	700	Hydrolysis of amide	351.1695	Non-Mutagenic (0.35)

	D4	Q	700	Hydrolysis of amide	102.0681	Non-Mutagenic (0.35) Hepatotoxic (0.66)
	D5	D1	927	Oxidative cleavage of alpha-functionalised carboxylic acid or amide	421.2114	Mutagenic 0.61 Immunotoxic (0.65)
	D6	D1	900	Cleavage of acyclic hemiaminal, hemiacetal or hemimercaptal	250.0855	Non-Mutagenic (0.30)
	D7	D1	900	Cleavage of acyclic hemiaminal, hemiacetal or hemimercaptal	201.1365	Non-Mutagenic (0.30)
	D8	D2	925	Oxidation of N-alkyl amide to hemiamidal	421.2114	Mutagenic 0.61 Immunotoxic (0.65)
	D9	D2	900	Hydrolysis of imide or thioimide	335.1746	Non-Mutagenic (0.30) Immunotoxic (0.65)

	D10	D2	900	Hydrolysis of imide or thioimide	88.0524	Non-Mutagenic (0.0)
	D11	D2	900	Hydrolysis of imide or thioimide	321.159	Non-Mutagenic (0.0)
	D13	D3	700	Hydrolysis of amine	252.1011	Non-Mutagenic (0.16)
	D14	D3	700	Hydrolysis of amine	117.079	Non-Mutagenic (0.04) Hepatotoxic (0.51)
	D15	D3	700	Oxidation of alpha-carbonyl amine or alcohol	349.1539	Non-Mutagenic (0.36)
	D17	D3	687	Oxidation of primary or secondary amine to imine	349.1539	Non-Mutagenic (0.43)

The summary of the drug degradation prediction study is given in the following tables.

Table 6.5: Drug degradation prediction for VAL

Matrix- Valsartan	Total degradants predicted by Zeneth
General	9

Acid Degradation - 1 N HCl	14
Base Degradation – 1 N NaOH	12
Oxidative Degradation – 10% H_2O_2	9
Thermal Degradation - 80°C	5
Photo-degradation	3

Table 6.6: Drug degradation prediction for NBL

Matrix- Nebivolol hydrochloride	Total degradants predicted by Zeneth
General	4
Acid Degradation - 1 N HCl	3
Base Degradation – 1 N NaOH	4
Oxidative Degradation – 10% H_2O_2	3
Thermal Degradation - 80°C	0
Photo-degradation	0

Table 6.7: Drug degradation prediction for SAC

Matrix- Sacubitril	Total degradants predicted by Zeneth
Sacubitril – General (hydrolysis/neutral)	15
Acid Degradation - 1 N HCl	40
Base Degradation – 1 N NaOH	30
Oxidative Degradation – 10% H_2O_2	2
Thermal Degradation - 80°C	0
Photo-degradation	6

Table 6.8: Drug degradation prediction for NBL-VAL mixture

Matrix- Nebivolol-Valsartan mixture	Total degradants predicted by Zeneth
Nebivolol-Valsartan (as excipient)	25
Valsartan-Nebivolol (as excipient)	13
Combine salt form	38

Table 6.9: Drug degradation prediction for SAC-VAL mixture

Matrix- Sacubitril-Valsartan mixture	Total degradants predicted by Zeneth
Sacubitril-Valsartan (as excipient)	15
Valsartan-Sacubitril (as excipient)	9
Combine salt form	24

Table 6.10: Drug degradation prediction for Drug: (if NDMA & NDEA impurity present in drug sample)

Matrix	Number of Degradants Predicted by Zeneth	Number of Mutagenic/ genotoxic impurities predicted by TEST
Valsartan	**9**	**2**
Nebivolol hydrochloride	**4**	**1**
Sacubitril	**15**	**0**
Nebivolol - Valsartan	**38**	**16**
Sacubitril- Valsartan	**24**	**2**
Nebivolol hydrochloride + NDMA	**25**	**1**
Valsartan + NDMA	**9**	**3**
Sacubitril + NDMA	**15**	**1**
Nebivolol - Valsartan + NDMA	**38**	**17**
Sacubitril- Valsartan + NDMA	**24**	**2**
Nebivolol hydrochloride + NDEA	**25**	**1**
Valsartan + NDEA	**9**	**3**
Sacubitril + NDEA	**15**	**1**
Nebivolol-Valsartan + NDEA	**38**	**17**
Sacubitril- Valsartan + NDEA	**24**	**2**

NDMA: N-Nitrosodimethylamine **NDEA:** N-Nitrosodiethylamine

Major findings based on Zeneth's drug degradation prediction were discussed below.

Zeneth drug degradation prediction revealed that the degradation formation in VAL is primarily attributed due to the hydrolysis of amide/imide or thioamide reactions, with oxidation

playing a secondary role. The instability of NBL, on the other hand, is mainly due to oxidation reactions. In the case of SAC-VAL, oxidative reactions followed by hydrolysis are the main contributors to its instability. Meanwhile, NBL-SAC is highly prone to degradation through oxidation, followed by hydrolysis and O-acylation of alcohol reactions.

The parent Q indicates the primary degradants of the respective drug matrix. The comparison between first-line degradants predicted by the zeneth and actual degradants observed in the chromatographic analysis were compared. It was concluded that the number of first-line degradants and actual degradants formed were nearly the same in number. SAC as a single drug product is more susceptible to degradation compared to VAL and NBL. However, in combination with VAL, SAC exhibits greater stability than in its API form. The NBL-VAL mixture is more prone to degradation compared to their individual APIs. Fixed dose combinations have a higher likelihood of producing degradation products compared to single APIs.

The presence of genotoxic impurities in the drug product does not significantly impact the formation of more degradants for VAL, SAC, NBL-VAL, and SAC-VAL. The presence of genotoxic impurities in the drug product may increase the number of toxic degradants compared to non-genotoxic drug degradants. It was observed in the NBL-SAC stability study, the concentration/amount difference in the drug can mask the degradation formed during chromatographic separation due to area and peak height difference. The toxicity prediction and confirmation of genotoxicity through the Ames test revealed that the degradants could be genotoxic in nature this will emphasize the need for toxicity assessment of the drug and its DPs.

Summary and Conclusion

Robust, specific, and easy-to-use QbD-enabled stability-indicating UFLC methods were developed to investigate the stability behaviour of antihypertensive drugs, VAL and NBL, as well as their combination (NBL-VAL and SAC-VAL). The degradants identified in the chromatographic study were characterised with the aid of LC-MS/MS. A drug degradation prediction study was conducted on all matrices. The toxicity of all characterized degradants was predicted using TEST and predicted toxic degradants were isolated via PLC. Subsequently, the

predicted toxicity of degradants was confirmed by performing the genotoxicity study (Ames test). The proposed UFLC methods can accurately and specifically detect and measure anti-hypertensive drugs and their combinations, making them suitable for both regular and stability analyses.

This study will provide analysts and researchers with valuable insights for improving impurity profiling by enabling them to design a more precise plan. The prediction of drug degradation will aid in evaluating the stability of both the drug and drug samples prior to conducting the actual stability study. By utilizing *in silico* degradation prediction and toxicity assessment, the safety and stability of the drug sample and drug product can be assessed. Additionally, this study can contribute to the development of a targeted stability-indicating analytical method, which will provide a comprehensive understanding of impurity/degradation profiling. Furthermore, the stability prediction can be extended to drug-drug compatibility studies and drug-excipient compatibility studies, facilitating the design of more stable and safe drug formulations.

Consequently, drug degradation prediction, comparison with actual degradation, development of the targeted stability-indicating analytical method, toxicity assessment of degradants, and confirmation of the predicted toxicity will significantly reduce the risk of drug instability and eventually drug recall. Therefore, we can serve safe and stable drug formulation to society. Furthermore, the reaction pathways, fragmentation pattern, and degradant structure will help to target the most susceptible area/site/molecule/functional group/bond of drug structure, which is responsible for degradation or which is more unstable/reactive. The same can be protected by complexation/derivatisation so it will help in drug design and to develop the next-generation molecules, which will be more effective, safe, and stable.

Publication details

Name of the author/s	Title of the article/research paper	Name of the Journal	Volume / Page no.	Year	Impact Factor
Mr. Madhav Shelke, Dr. Shirish Deshpande, Dr. Sanjay Sharma	Quinquennial Review of Progress in Degradation Studies and Impurity Profiling: An Instrumental Perspective Statistics	Critical Reviews in Analytical Chemistry	50(3) 226-253	2020	**6.535**
Mr. Madhav Shelke, Dr. Chandrakant Bonde, Dr. Shirish Deshpande, Dr. Ritesh Bhole	Drug degradation prediction, *in-silico* toxicity assessment and development of stability-indicating, Quality by Design enabled UFLC method for Sacubitril-Valsartan	Russian Journal of Bioorganic Chemistry	50 1-18	2023	**1.254**
Mr. Madhav Shelke, Dr. Chandrakant Bonde,	Exploring the Stability Behavior of Valsartan in the Presence of Genotoxic Impurity: An in-silico Study for Predicting Drug Safety and Quality Control	Biomedical Chromatography	Under review	--	**1.911**
Mr. Madhav Shelke, Dr. Chandrakant Bonde,	Exploring Drug Safety and Stability Behavior of Nebivolol-Valsartan: An *In-Silico* Study for Quality Control and toxicity evaluation	Drug and Chemical Toxicology	Under review	--	**2.597**

List of Conferences / Seminars (Webinar) / Workshops attended

Name of the conference/seminar/webinar	Year	Organizing Body / Inst.
One-Week National Level Workshop on "Drafting Research Publication, Funding Proposal & Ethical Conduct of Research"	2022	Department of Pharmaceutical Sciences, RTMNU, Nagpur
NDMA Impurity in Medicine: A Source of Bewilderment for the Patients	2020	Society of Pharmaceutical Sciences and Research
International Conference on Recent Advancements in Sciences and Technology	2020	Glocal University, Saharanpur, UP
International Conference on Current Trends in Pharmaceutical Industry	2020	Maharishi Markandeshwar University, Sadopur-Ambala
Choosing the Best UPLC System for Your Application Needs: Autosampler Designs in LCs	2019	SelectScience and Waters Corporation
Two Days Workshop on QbD & Hands-on Training on DOE	2017	SVKM's NMIMS SPTM, Shirpur & ZIM Laboratories Ltd.

References:

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- Bharate SS. Critical Analysis of Drug Product Recalls due to Nitrosamine Impurities. J. Med. Chem. **(2021)** 64: 2923–2936.
- Ali MA, Hemingway R, and Ott MA. *In silico* drug degradation prediction. Methods Pharmacol. Toxicol. **(2018)** 53–73.
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- Chodankar RS and Mahajan AA. Characterization and In-silico toxicity prediction of degradation products of felbamate. Futur. J. Pharm. Sci. **(2021)** 7: 1–10.
- Farrukh MJ, Tariq MH, Malik O, and Khan TM. Valsartan recall: global regulatory overview and future challenges. Ther. Adv. Drug Saf. **(2019)** 10: 1–4.
- User's Guide for T. E. S. T. (Toxicity Estimation Software Tool) Version 5.1
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